TRANSMEMBRANE SIGNAL TRANSDUCTION: STRUCTURE, MECHANISMS, REGULATION OF EVOLUTION

Organizers: Merl F. Hoekstra, Tony Hunter, Heidi Hamm, Jim Hicks, Tony Pawson, Edward A. Dratz and Alfred Wittinghofer

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Transmembrane Signal Transduction: Structure, Mechanisms, Regulation and Evolution

Receptor Signalling: An Overview

I 001 HETEROMERIC KINASE RECEPTORS FOR THE TGF-β FAMILY, Joan Massagué, Liliana Attisano, Juan Cárcamo, Fernando López-Casillas, Francesc Ventura, Frances M. B. Weis, Rotraud Wieser and Jeffrey L. Wrana, Cell Biology and Genetics Program and Howard, Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

The TGF-β-related cytokines, one of the largest groups of growth and differentiation factors, interact with pairs of membrane proteins known as receptor types I and II that constitute a family of transmembrane protein serine/threonine kinases. Although the type II receptors for TGF-β, activin and bone morphogenetic proteins bind ligand with high affinity and contain a cytoplasmic signaling structure, most of the evidence available so far indicates that these receptors are unable to signal alone; they require the presence of an appropriate type I receptor with which they form a complex. Moreover, type I receptors require type II in order to bind ligand. Various members of the transmembrane serine/threonine kinase family from mammals and *Drosophila* have now been identified as type I receptors from TGF-β, activin or BMP. Pairwise combinations of receptor types I and II dictate the binding and signaling properties of the resulting complexes. For example, the TGF-β type I receptor TβR-I and the activin type I receptor ActR-IB bind the corresponding ligands in concert with the respective type II receptors, TβR-II and ActR-II. The kinase domains of TβR-II and ActR-II are very divergent, but the kinase domains of TβR-I and ActR-IB are highly related and both mediate the same set of responses including cell growth arrest. Another type I receptor, ActR-I (a.k.a. Tsk 7L or SKR1) forms a functional activin receptor complex with ActR-II, and interacts weakly with TGF-β and TβR-II. However, the related receptor TSR-I can bind TGF-β in concert with TβR-II and activin in concert with ActR-II, mediating as yet unknown responses. Thus, the TGF-β family receptor structure that is emerging from these studies is that of a complex containing two distantly related transmembrane serine/threonine kinases that cooperate to define the ligand binding and signaling specificity of the complex. This basic structure appears to be conserved from mammals to *Drosophila*.

TGF-β access to signaling receptors is controlled by betaglycan (the type III receptor), a membrane-anchored proteoglycan whose core protein binds TGF-β. Betaglycan has a dual function: its membrane-anchored form serves to present TGF-β directly to receptor II, enhancing binding and cell responsiveness to TGF-β. In contrast, the ectodomian of betaglycan released into the medium acts as an antagonist of TGF-β binding to membrane receptors.

I 002 CYTOKINE SIGNALLING AND TARGET GENES
Tadatsugu Taniguchi, Institute for Molecular and Cellular Biology, Osaka University, Suita-shi, Osaka 565, Japan

Cytokines regulate cell growth in a positive or negative manner by inducing their respective target genes. We have been focusing on two cytokine systems, the IL-2 and IFN systems. IL-2, one of the best studied cytokines, is a potent growth stimulatory factor for lymphocytes, whereas the IFNs are well known "negative growth factors", which inhibit the growth of various cell types including hematopoietic cells.

Recently, a number of cytokine receptors including IL-2 receptor(IL-2R), have been analysed. Many of these receptors shave significant homology and constitute a novel family of receptors, characterized by four conserved cysteins and the sequence WSXWS (the WS motif). One interesting feature of this new cytokine receptor family is that its members lack the intrinsic protein tyrosine kinase domain that is the hall mark of other growth factor receptors. The IL-2R is unique in that it is made up of at least three distinct membrane components: the α chain (IL-2Rα), the β chain (IL-2Rβ), and the γ chain (IL-2Rγ). We and others have provided evidence for the requirement of the cytoplasmic regions of IL-2Rβ and IL-2γ, but not IL-2Rα, in the IL-2-induced signalling to cell interior. The IL-2Rβ chain has been shown to interact, both physically and functionally, with the nonreceptor tyrosine kinase Src family members ($p56^{lck}$, $p59^{fyn}$, $p53/56^{lyn}$). Likewise, other members of the cytokine receptor family seem to utilize another nonreceptor tyrosine kinase members. We have identified several target genes which would mediate IL-2-induced cell proliferation of a hematopoietic cell line. Evidence suggests that the IL-2Rβ-Src kinase pathway is linked to $p21^{ras}$ and subsequently to *c-fos*, *c-jun* activation. On the other hand, another signalling pathway (s) emanating from IL-2Rβ, IL-2Rγ heterodimer, but not linked to the Src kinase pathway, leads to activation of genes such as *c-myc*, *bcl-2* and others, followed by the progression of the cell cycle. We will present our recent results on the role of these target genes in cell growth control.

During the study of the IFN system regulation, we identified two transcription factors, IRF-1 (activator) and IRF-2 (repressor). IRF-1 is IFN-inducible and evidence suggests that IRF-1 is in fact a target gene critical for the IFN functioning as negative regulator of cell growth. More recently, we have provided evidence that IRF-1 and IRF-2 manifest anti-oncogenic and oncogenic potentials, respectively. In order to gain further insights on the role of the IRFs, mice deficient in either IRF-1 or IRF-2 or both have been generated. We will present our recent results on the role of these two IRFs in the context of cell growth regulation.

The work has been carried out partly in collaboration with R. Perlmutter (University of Washington), C. Willman (University of New Mexico), T. Mak (Ontario Cancer Center) and their colleagues.

Heptahelical Receptor Signalling

I 003 GENETIC REGULATION OF OLFACTORY SIGNALING PATHWAYS, Randall R. Reed, Janine A. Davis, Irene C. Griff, Pankaj Qasba Karen A. Schrader and Robert Y.L. Tsai , The Johns Hopkins University, Department of Molecular Biology and Genetics, Baltimore, Maryland

The mammalian olfactory system displays remarkable sensitivity and specificity in the detection of odorant molecules. Recent biochemical and electrophysiological experiments have established that the initial events in the conversion of the external stimulus into the signal received by the brain are mediated by a G-protein signalling cascade. Each of the components of this cascade appear to be expressed at high levels in the sensory neurons of the olfactory system. We have identified a transcription factor, Olf-1, that appears to regulate the coordinate expression of these olfactory signaling components and are examining the patterns of expression of this protein and the additional transcription factors which may control Olf-1 expression. The ability of the mammalian olfactory system to discriminate among structurally related compounds is thought to result from a family of related receptors each expressed in a subset of the sensory neurons. Recent work has suggested that the odorant receptor family consists of 500-800 genes. Immunohistochemical and in situ hybridization experiments reveal that only a small fraction of the receptor cells express any particular receptor gene. The organization of the genes encoding these receptors and the mechanisms that direct their expression to subsets of the sensory neurons is currently being examined.

CA++ and Lipid Signalling Pathways

I 004 **PI 3-KINASE SIGNALING PATHWAY REGULATES PROTEIN SORTING IN THE YEAST SECRETORY PATHWAY**

Jeffrey H. Stack, Kaoru Takegawa, Peter V. Schu and Scott D. Emr, Division of Cellular and Molecular Medicine, Howard Hughes Medical Institute, University of California-San Diego, School of Medicine, La Jolla, CA 92093-0668

Genetic analysis of protein sorting to the yeast lysosome-like vacuole has permitted the isolation of a large number of *vps* (vacuolar protein sorting) mutants which missort and secrete vacuolar hydrolases. *vps*15 and *vps*34 mutants appear to form a unique subset among the *vps* mutant collection. They share a common set of growth, morphology and protein sorting defects. The *VPS*15 gene encodes a membrane-associated protein kinase (1,455 aa) that is myristoylated at its N terminus. The *VPS*34 gene encodes a phosphatidylinositol 3-kinase (PI 3-kinase) that is homologous to the catalytic subunit (p110) of mammalian PI 3-kinase. Changes in residues of Vps15p as well as Vps34p that are conserved among protein kinases and lipid kinases, respectively, inactivate each kinase. The mutant cells are *ts* for growth and exhibit extreme defects in vacuolar protein sorting. The vacuolar hydrolase carboxypeptidase Y (CPY) is secreted by these mutant cells as the Golgi-modified precursor form of CPY (p2CPY). Overexpression of the Vps34 protein suppresses the growth and protein sorting defects exhibited by a Vps15 kinase domain point mutant indicating that the Vps15 protein kinase functionally interacts with the Vps34 lipid kinase.. A direct association between the Vps15 and Vps34 proteins has been demonstrated using both native immunoprecipitation and chemical cross-linking experiments. Activation of the Vps34 PI 3-kinase requires an active Vps15 protein kinase. Extracts from Vps15 kinase domain point mutants contain 10-fold lower levels of PI 3-kinase activity than equivalent extracts from wild-type cells. Vps15 protein kinase activity may itself be regulated by direct interaction with the cytoplasmic tail domains of particular transmembrane receptors, such as the CPY-specific sorting receptor (coded for by the *VPS*10 gene). In the Golgi lumen, ligand (CPY) binding to the receptor could result in the sequential activation of the Vps15 and Vps34 kinases. In such a model, the Vps10, Vps15 and Vps34 proteins effectively act as components of a signal transduction complex which converts the signal received by the CPY receptor into a second messenger (PI 3-phosphate) that then could trigger the action of as yet unknown effector proteins that direct protein sorting to the vacuole (e.g., vesicle coat proteins).

1) Schu, P. V., K. Takegawa, M. J. Fry, J. H. Stack, M. D. Waterfield and S. D. Emr. 1993. **Science**, 260: 88-91.
2) Stack, J. H., P. K. Herman, P. V. Schu, and S. D. Emr. 1993. **EMBO J.**, 12: 2195-2204.
3) Vida, T. A., G. Huyer and S. D. Emr. 1993. **J. Cell Biol.**, 121: 1245-1256.

Receptor Structure

I 005 **PROBING AND DESIGNING GROWTH HORMONE-RECEPTOR INTERACTIONS,**

James. A. Wells[1], Germaine Fuh[1], Henry Lowman[1], Tim Clackson[1] and Brian Cunningham[1], [1]Department of Protein Engineering, 460 Pt. San Bruno Blvd., So. San Francisco, CA 94080.

Human growth hormone (hGH) binds to either the growth hormone or the prolactin receptor. These receptors contain a single transmembrane and extra cellular domains are homologous to a number of other cytokine receptors. Scanning mutational analysis and biophysical studies showed that hGH contains two binding sites (called Sites 1 and 2) for the extra cellular domain of its receptor (called the hGHbp). Moreover, the receptor binds these sites sequentially, first at Site 1 then at Site 2, to form an hGH(hGHbp)2 complex. Studies on whole cells show that receptor dimerization is required to activate the receptor and induce cell growth. Based upon the sequential receptor dimerization mechanism it has been possible to build potent antagonists to the hGH and prolactin receptor by making hGH analogs that bind at Site 1 but not at Site 2.

A 2.8 Å x-ray structure of the complex (de Vos, A.M., Ultsch, M. and Kossiakoff, A.A. (1992) Science *255*, 306) confirmed the stoichiometry of the complex and revealed the molecular details of the interactions. Using a biochip device called BIAcore*TM* (from Phamacia) we investigated the hormone binding mechanism in much greater detail. These studies showed that of 31 contact residues, 15 have virtually no effect on binding when converted to alanine. Of the remaining 15, 7 can account for >80% of the binding free energy. Thus, the functional epitope (defined by alanine-scanning) is considerably smaller than the contact epitope (defined by x-ray crystallography).

Understanding the molecular basis for hormone action is fundamental to signal transduction. This research leads naturally to the generation of hormone agonists and antagonists with improved pharmocologies that may be of clinical use. Ultimately, we believe such studies will be instrumental in the rational design of small molecule mimics of peptide hormones.

Regulation of Chromosome Mechanics

I 006 THE REGULATION OF DNA REPAIR PATHWAYS BY CASEIN KINASE I, Merl Hoekstra, Namrita Dhillon and Anthony DeMaggio, ICOS Corp., 22021-20th Ave. S.E., Bothell, WA 98021

A wide variety of functions have been identified for repairing genotoxic damage. Such functions include the enzymatic components involved in DNA repair while other functions include regulatory protein such as protein kinases. In *S. cerevisiae*, the *RAD* gene products are required for repairing UV and X-ray induced lesions and several *CDC* proteins have been implicated in DNA repair. We have identified genes in *S. cerevisiae* and *Sc. pombe* involved in repairing DNA double-strand breaks. The bakers' yeast *HRR25* gene was identified through its mutant phenotypes, which resulted in *S. cerevisiae* strains that are unable to tolerate persistent DNA double-strand breaks. *Hrr25-1* strains are sensitive to agents that cause DNA double-strand breaks, resistant to UV, proficient for mitotic recombination, and unable to proceed through meiosis. Deletion of *HRR25* results in cell cycle defects and aberrant cellular morphology. *HRR25* encodes a protein kinase domain and Hrr25p-dependent activity has been detected.

We have isolated and characterized two gene from *Sc. pombe*, called *hhp1+* and *hhp2+*, which are functionally homologous to *HRR25*. Protein kinase activity has been detected for hhp1 and, like Hrr25p, the activity is multi-specific for serine, threonine, and tyrosine residues. Mutant analysis has revealed that*Sc. pombe hhp1Δ* and *hhp2Δ* mutants are sensitive to both physical and chemical agents that cause DNA damage. We also observe spontaneous phenotypes in *hhp1Δ* and *hhp1Δ hhp2Δ* strains. These phenotypes include an aberrant microscopic morphology, impaired growth rate, and altered nucleus segregation patterns. In depth analysis of the DNA repair defects in these mutants has revealed a differential response to various damaging agents. The lethality seen in *hhp* mutants is not simply due to the loss of a checkpoint control in the cell cycle but is a direct consequence of the inability to repair DNA. The differential responses provide evidence for the existence of two distinct DNA repair pathways that depend upon the form of DNA lesion.

HRR25, *hhp1+* and *hhp2+* protein kinases are most closely related to mammalian casein kinase I. Consistent with its similarity, Hrr25p activity *in vitro* is similar to classically-defined mammalian CKI activity. Based on their amino acid sequence conservation and *in vitro* properties, these genes belong to the Casein Kinase I subfamily of protein kinases and reveal a role for this protein kinase subfamily in regulating DNA metabolism.

I 007 CELL CYCLE CONTROL AND GENETIC INSTABILITY, Geoffrey M. Wahl, Aldo Di Leonardo, Steven P. Linke, Yuxin Yin, The Salk Institute, San Diego, CA.

The conversion of a normal cell into a cancer cell involves the accumulation of multiple genetic lesions. Gene amplification, a marker for genetic instability, is often initiated by chromosome breakage. Since amplification occurs at a high frequency in tumor cells but is not detectable in normal cells, it is likely that control mechanisms exist to minimize the cycling of normal cells with damaged genomes. We assayed for cells that gained the capacity to undergo gene amplification to elucidate the gene products involved in such controls. The tumor suppressor p53 has been implicated in one such control pathway by the activation of a G1 checkpoint leading to arrest of the cells in response to irradiation and certain chemotherapeutic agents. We studied the role of p53 in amplification of the CAD gene by selection with PALA. Mutations that interfere with the correct functioning of the p53 tumor suppressor enabled the selection of PALA resistant cells harboring amplified CAD genes, and restoring wild type p53 function prevented PALA selected gene amplification from occurring. Cytogenetic and *in situ* assays for DNA damage indicated that PALA produces DNA damage in $p53^-$ cells, making them competent for gene amplification. We propose that this occurs because the cells continue cycling through multiple S phases with severely depleted DNA precursor pools. We compared the cell cycle kinetics of human cells treated with g-rays and the *de novo* UMP synthesis inhibitors PALA and pyrazofurin. Asynchronous normal human fibroblasts (containing wild-type p53) arrested in G1 in response to both PALA and irradiation. By contrast, cells with mutant p53 or lacking p53 expression continued to cycle under these conditions. These data are consistent with DNA damage being one trigger for p53-induced G1 arrest. However, the following data suggest the existence of a second trigger. Normal human cells synchronized by serum starvation in G0/G1 and released in the presence of PALA either remained in G1 or traversed through only one cycle and arrested in the next G1. No detectable single-strand nicks or double-strand breaks were detected under these conditions. Furthermore, the g-radiation-induced G1 arrest of normal cells appeared to be irreversible, even at low doses. By contrast, the PALA-induced G1 arrest was readily reversible, even at high doses, by removing the drug or by adding uridine. Collectively, these data suggest at least two mechanisms for a p53-induced G1 arrest. We propose that p53 is part of a pathway that responds to nucleotide pool imbalance by eliciting a reversible G1 arrest, and that it responds to DNA breakage by eliminating damaged cells from a population through an irreversible G1 arrest eventually leading to cell death.

SH2/SH3

I 008 PROTEIN-TYROSINE KINASE SIGNALING VIA SH2 AND SH3 DOMAINS, Lewis Cantley, Harvard Medical School and Beth Israel Hospital, Boston, MA 02115.

Protein-tyrosine kinases find their downstream targets by multiple mechanisms. Autophosphorylation of receptor tyrosine kinases results in recruitment of specific SH2 domain-containing proteins to the receptors via sequence-specific phosphotyrosine-SH2 domain interactions. Conformational changes due to phosphotyrosine-binding to the SH2 domains induce conformational changes in the downstream targets, such as PtdIns 3-kinase, that regulate enzymatic activities. Non-receptor protein-tyrosine kinases use intrinsic SH2 and SH3 domains to form contacts with downstream targets such as PtdIns 3-kinase. We have recently developed a peptide library technique that allows us to determine the optimal phosphotyrosine peptide sequence for individual SH2 domains (Songyang et al, 1993, Cell 72, 767). The optimal motifs for 25 different SH2 domains have now been determined by this procedure. These motifs suggest likely targets for individual SH2 domains *in vivo*, some of which have been confirmed. We have also developed a similar technique for determining the optimal motif for peptide substrates of protein kinases. This procedure has been used to investigate likely targets of receptor and non-receptor protein-tyrosine kinases. It has also been used to determine optimal motifs for certain protein-serine kinases involved in cell growth regulation. The motifs provide important clues about how protein kinase target specific proteins in vivo.

I 009 CRYSTAL STRUCTURES OF SH2 AND SH3 DOMAINS, John Kuriyan[1,2], Chi-hon Lee[2], Dorothea Kominos[2], Gabriel Waksman[2], Xiaodong Wu[2], T.S.R. Krishna[2], David Cowburn[2] and Steven E. Shoelson[3]. [1]Howard Hughes Medical Institute, [2]The Rockefeller University, 1230 York Avenue, New York, NY 10021 and [3]Joslin Diabetes Center, Harvard Medical School, Boston.

The crystal structure of the N-terminal SH2 domain of the protein tyrosine phosphatase SHPTP1D has been determined in the presence and absence of phosphopeptides. Although this SH2 domain is quite distant in sequence from the *src* SH2 domain, the crystal structures of the two SH2 domains are closely superimosable, with the major changes in structure being localized to the peptide binding regions. A notable feature of the phosphatase SH2 domain is the absence of an otherwise conserved arginine residue (Arg aA2) that in src and lck has been seen to interact with the phosphate group and the aromatic ring of the phosphopeptide. The crystal structure of one of the SH3 domains of the *nck* oncogene product has been determined, and its structure will be discussed.

I 010 **SH2 AND SH3 DOMAINS CONTROL PROTEIN INTERACTIONS IN SIGNAL TRANSDUCTION,** **Tony Pawson, Ph.D., Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave., Toronto, Ontario M5G 1X5 Canada**

SH2 and SH3 domains are protein modules found in a variety of cytoplasmic signalling proteins, that act as targets of receptor protein-tyrosine kinases. The autophosphorylation of growth factor receptors creates high affinity binding sites for SH2 domains, that directly recognize phosphotyrosine and adjacent residues. Hence receptor autophosphorylation acts as a molecular switch to induce the association of receptor tyrosine kinases with their cytoplasmic targets. This association can recruit signalling proteins to the membrane, and can enhance their phosphorylation on tyrosine.

SH3 domains apparently recognize proline-rich motifs. A number of adaptor proteins possess only SH2 and SH3 domains, and may therefore couple tyrosine kinases to downstream targets with such SH3-binding sites. A specific example of this is provided by the Drk/Grb2 protein, which couples receptor tyrosine kinases to Sos, a Ras guanine nucleotide exchange factor, and thereby regulates activation of the Ras pathway.

A second example of an SH3-mediated interaction is provided by the Src SH3 domain, which directly contacts proline-rich motifs in the p85 subunit of phosphatidylinositol 3'-kinase. The p85 SH3 domain can itself interact with these sites. These results indicate that SH3 domains are involved in the regulation of p85 function, and in the association of PI 3'-kinase with the v-Src tyrosine kinase.

Cell Contact Signalling

I 011 **REGULATION ON TYROSINE PHOSPHORYLATION BY THE PLATELET INTEGRIN, $\alpha_{IIb}\beta_3$,** J.S. Brugge[1], E.A. Clark[1], L. Lipfert[1,2], B. Haimovich[3], M.H. Ginsberg[4], J.E.B. Fox[5], S.S. Shattil[2], [1]ARIAD Pharmaceuticals, Inc., Cambridge, MA 02139. [2]Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104. [3]Department of Surgery, Univeristy of Medicine and Dentistry of New Jersey, New Brunswick, NJ 08903. [4]Scripps Research Institute, La Jolla, CA. [5]Childrens Hospital Oakland Research Institute, Oakland, CA 94609.

We have previously shown that fibrinogen binding to its integrin receptor, $\alpha_{IIb}\beta_3$, is required for thrombin-induced tyrosine phosphorylation of multiple platelet proteins. The platelet system has been valuable in dissecting the intracellular signaling events that transduce integrin-regulated changes in cell behavior. We have found that $\alpha_{IIb}\beta_3$ regulated tyrosine phosphorylation could be separated into two distinct events: 1) the phosphorylation of p140 and several proteins of M_r 50-72 Kd (p50-72), which is induced by dimerization of integrin receptors with fibrinogen, and 2) the phosphorylation of p95/97 and the protein tyrosine kinase, $p125^{FAK}$, which is dependent on fibrinogen-induced platelet aggregation and a second costimulatory event. The induction of tyrosine phosphorylation of all of these proteins was inhibited in cytochalasin D treated platelets, suggesting that actin-dependent cytoskeletal complexes may couple the integrins with tyrosine kinases and their substrates. At least three classes of tyrosine protein kinases appear to participate in these events: $p125^{FAK}$, which is activated following platelet aggregation; $pp60^{src}$, which is activated independent of $\alpha_{IIb}\beta_3$ but redistributes to integrin-regulated cytoskeletal complexes after platelet aggregation, and Syk, which is phosphorylated on tyrosine following dimerization of $\alpha_{IIb}\beta_3$ by fibrinogen.

I 012 **THE ROLE OF ICAMS IN LYMPHOCYTE ACTIVATION AND ADHESION,** W. Michael Gallatin, Denise Hoekstra, Stephen Rosenman, Mary Poss, Gary Peterman, Tim Axtelle, Ray Fox, and James B. Hicks, ICOS Corporation, 22021 20th Avenue SE, Bothell, WA 98021.

TcR mediated T lymphocyte activation is supported by accessory molecules which provide adhesive and/or signaling functions. We recently described an adhesion molecule, ICAM-R (ICAM-3), an LFA-1 counterreceptor constitutively expressed on both T cells and accessory cells. To demonstrate a role for ICAM-3 in T cell activation, we examined the effects of a series of ICAM-3 specific Mabs in several in vitro models. Crosslinking ICAM-3 was costimulatory with TcR crosslinking in inducing CD4+ T cell activation and proliferation. Crosslinking ICAM-3 alone induced the expression of activation associated antigens such as CD69, but not proliferation. Specific ICAM-3 Mabs are capable of blocking the induction of accessory cell dependent T cell activation by the super antigen, SEA, or alloantigen. Pretreatment of either accessory or $CD45RO^+$ T cells, but not $CD45RA^+$ T cells, impaired the SEA response. These results suggest that both T cell and accessory cell ICAM-3 may play a fundamental role in modulating T cell activation.

Differential activities of anti-ICAM-3 Mabs in qualitatively distinct functional assays (e.g. adhesion to recombinant proteins, T cell activation etc.) are correlated with the specific epitopes bound based on molecular genetic mapping studies performed with a more extensive series of Mabs and mutant ICAM-3 proteins. Additional experiments suggest that ICAM-3 promotes lymphocyte activation not only by passively promoting leukocyte-leukocyte adhesion, but also through direct connection with intracellular signal transduction pathways. We have, therefore, begun to examine the cytoplasmic consequences of ICAM-3 receptor engagement using genetic and biochemical techniques. The role of these in ICAM-3 signalling during T cell activation will be discussed.

Receptor Kinases

1013 THE ROLE OF THE *DROSOPHILA* EGF RECEPTOR HOMOLOG (DER) AND ITS LIGAND, SPITZ, IN ESTABLISHMENT OF EMBRYONIC DORSAL-VENTRAL POLARITY, Ben-Zion Shilo, Erez Raz and Ronen Schweitzer. Department of Molecular Genetics and Virology, Weizmann Institute of Science, Rehovot 76100, Israel.

The embryonic phenotype resulting from mutations in the *Drosophila* EGF receptor homolog (DER) is complex, since the receptor is required multiple times during embryogenesis. Utilizing a temperature-sensitive allele the phenotype could be dissected and the earliest roles identified. We found that at 3.5 hours of embryonic development, DER is essential for establishing the identity of cells within the ventral ectoderm. In the absence of DER activity at this phase, alterations in cell fate are observed: Ventral cells acquire more dorsal fates, and the central nervous system, created by neuroblasts delaminating from the ventral ectoderm, is severely disrupted. DER is thus a crucial element in establishing or maintaining the signals generating a spectrum of ventral cell fates in the embryonic ectoderm of *Drosophila* . The ventralizing effect of DER appears to function later than that of the dorsalizing *dpp* pathway, and the spatial overlap between them is minimal.

Some aspects of the mutant ectodermal and CNS phenotypes of the DER locus (*faint little ball, flb*) resemble the phenotype of mutations from the *spitz* group (including *spitz, Star, rhomboid* and *pointed*). Synergistic interactions between *flb* and *spitz* or *Star* mutations have indeed been demonstrated, suggesting that these genes participate in a common signaling pathway. The deduced structure of the spitz protein displays homology to TGFα, one of the ligands of the vertebrate EGF receptor, and to lin-3, the putative ligand of the *C. elegans* EGF receptor homolog. We have shown that the secreted form of the spitz protein triggers the tyrosine autophosphorylation activity of DER expressed in *Drosophila* Schneider cells, as well as the association of DER with the Drk protein containing SH2 and SH3 domains. Elucidating the molecular basis for the participation of the *spitz* group proteins in DER signaling, is crucial for understanding the regulation of the pathway. The possibility that other members of the *spitz* group which encode membrane proteins, most notably rhomboid, facilitate or enhance the activation of DER by spitz is being examined.

Receptor: G Protein Interactions

1014 GENETIC ANALYSIS OF G PROTEIN FUNCTION IN YEAST, Malcolm Whiteway, Ekkehard Leberer, Karen Clark, Daniel Dignard, Doreen Harcus, Linda Hougan and David Y. Thomas, Biotechnology Research Institute, 6100 Royalmount Avenue, Montreal, Quebec, Canada, H4P 2R2.

The *STE4* and *STE18* genes of *S. cerevisiae* encode the β and γ subunits of a G protein required for the activation of the yeast mating response signal transduction pathway. Thus the situation in yeast contrasts with many of the well studied G protein mediated signaling pathways in higher cells, which use the α subunit of the G protein to activate the response. We have used saturation mutagenesis to investigate various aspects of the function of the Ste4 protein. Selection of mutant proteins which could interfere with signal transmission in the presence of wild type Ste4 protein defined two small domains apparently involved in effector activation. Suppression of these mutants by high copy plasmids led to the identification of the Ste20 kinase, a new component of the signal transmission pathway, which serves to couple the G protein to the downstream kinase cascade. Other Ste4 mutant proteins were identified which prevented proper interaction between Ste4 and the α subunit encoded by *GPA1*. These mutants defined a domain of the Ste4 protein required only for interaction with α; suppressors of this defect were selected that defined a residue in Gpa1 involved in α/β interaction. Thus genetic investigation of the G protein involved in the yeast pheromone response can provide general information about this important class of signaling molecules.

Interferon and Immune Cell Signalling

1015 SIGNAL TRANSDUCTION FROM CELL SURFACE TO NUCLEUS, James E. Darnell, Jr.,The Rockefeller University, New York, N.Y. 10021

Binding of IFN-α (interferon alpha) and IFN-γ (interferon gamma) to their cell surface receptors promptly induces tyrosine phosphorylation of latent cytoplasmic transcriptional activators, which we designate STATs (signal transducers and activators of transcription). IFN-α activates both Stat91 [the 91 kD protein] and Stat113 [the 113 kD protein] while IFN-γ activates only Stat91. The activated proteins then move into the nucleus and directly activate genes induced by IFN-α and IFN-γ. Somatic cell genetics experiments have demonstrated a requirement for tyrosine kinase 2(Tyk2) in the IFN-α response pathway and Jak2, a kinase with similar sequence, in the IFN-γ response pathway. We have investigated the tyrosine phosphorylation events on STAT and JAK proteins after treatment of cells with IFN-α, γ, and EGF. Stat91 is phosphorylated on tyrosine^{701} after cells are treated with IFN-α and EGF, just as we had earlier found after IFN-γ treatment. Correlated with these results, we find that Jak1 becomes phosphorylated on tyrosine after cells are treated with the same three ligands although each ligand is demonstrated to activate at least one other kinase. We propose therefore that Jak1 may be the enzyme that phosphorylates tyrosine^{701} in Stat91.

In addition to activation by the INFs we and collaborators (M. Gilman and H. Sadowski) have found that EGF and PDGR also lead to tyrosine phosphorylation of Jak1 and of Stat91. The exact composition of DNA binding factors induced under these condiditions is under study. These results imply that other ligands may use similar direct nuclear signalling mechanisms.

Finally, we have preliminary evidence for additional members of the STAT protein family and evidence that the STAT proteins can dimerize.

These findings lead to a general model for direct signal transduction from the cell surface to the nucleus that can explain the specificity inherent in the different responses to different polypeptide ligands.

1016 SIGNALING BY TNF RECEPTORS, David V. Goeddel[1], Mike Rothe[2], Louis A. Tartaglia[2] and Merrill Ayers[2], [1]Tularik, Inc., South San Francisco, [2]Genentech, Inc., South San Francisco.

The biological activities of tumor necrosis factor (TNF), a potent cytokine produced primarily by activated macrophages, are mediated by two specific cell surface receptors, TNF-R1 (55 kd) and TNF-R2 (75 kd). We have shown previously that both TNF receptors are active in signal transduction. TNF-R1 mediates signals for cytotoxicity and many other TNF activities in diverse cell types. In these systems TNF-R2 contributes to the cytotoxic signal that is initiated through TNF-R1 by facilitating the binding of TNF to TNF-R1 in a non-signaling mechanism. Demonstration of direct signaling by TNF-R2 has so far been restricted to a small subset of TNF activites in some lymphoid cells such as the stimulation of thymocyte proliferation and proliferation of the murine cytotoxic T-cell line CT6.

The signaling mechanisms of both TNF receptors have been examined by transfection based assays. Results obtained suggest that the aggregation of TNF-R1 intracellular domains, which are not associated in the absence of ligand, is an important component of the signal for cellular toxicity. Mutational analysis of the human TNF-R1 intracellular domain has defined a region near the C-terminus that transduces the signal for cytotoxicity. This "death domain" has a region of weak homology to the intracellular domain of the FAS antigen, which mediates a programmed cell death very similar to that signaled through TNF-R1. This result establishes a potential functional conservation between these two receptors. Surprisingly, when transfected into murine CT6 cells these two receptors elicit opposite biological activities: TNF-R1 activation induces cell proliferation whereas activation of the Fas antigen causes cell death.

An examination of TNF-R2 signaling has shown that in CT6 cells this receptor can activate the transcription factor NFκB and mediate the transcriptional induction of the GM-CSF gene. Interestingly, the GM-CSF gene is induced through activation of TNF-R1 in fibroblasts. A mutational analysis of the intracellular domain of the human TNF-R2 is presently underway.

1017 HOW DOES INTERFERON TICKLE TYK ? Laura Velazquez[1], Giovanna Barbieri[1], Knud E.Mogensen[2], Gilles Uzé[2], Marc Fellous[1] and Sandra Pellegrini[1]; [1]Institut Pasteur, INSERM U 276 Paris, France; [2]Institut de Génétique Moléculaire, CNRS, Montpellier, France.

Tyk2, JAK1 and JAK2 are the known members of a family of broadly expressed non-receptor protein tyrosine kinases characterized by a large amino terminal non-catalytic region, a kinase-like domain of unknown function and a tyrosine kinase domain. Recently, these proteins have been implicated in signalling pathways utilized by the superfamily of cytokine receptors. Interferon receptors, as all cytokine receptors, lack a catalytic domain in their cytoplasmic portions, but their engagement induces tyrosine phosphorylation of cytoplasmic transcription factors. With the help of cellular mutants defective in their responses to interferons, signalling components have been identified and studied. Thus, tyk2 was the first member of the JAK family to which a function could be assigned in signalling by IFN α/β as it rescues the defect of an IFN α/β unresponsive mutant. More recently, genetic evidence has implicated JAK2 in IFN γ signalling and JAK1 in both IFN α/β and IFN γ signalling pathways.

The tyk2 protein is found primarily in the cytosol with a minor fraction associated with the cell membrane. Short stimulation of cells with IFN α/β activates the protein : *in vivo* the protein becomes hyperphosphorylated and its *in vitro* kinase activity increases In stable transfectants expressing high tyk2 levels, the protein is highly phosphorylated and displays high kinase activity. However, the IFN response of these cells remains dependent on ligand binding.

Cells lacking tyk2 possess IFN α/β receptors with reduced ligand binding activity, suggesting that the kinase plays a role in the formation of receptor complexes. We have expressed altered forms of the tyk2 protein in the tyk2 deficient mutant and studied the phosphorylation and the kinase activity of the protein as well as the binding and IFN responsiveness of these cells. Deletion of either kinase domain abolishes kinase activity and *in vivo* induced phosphorylation. Recent observations suggest that ligand binding is restored in these transfectants and that, under certain conditions, signalling can be achieved. Our results point to a unique structural role of tyk2 in the formation of active receptor complexes.

1018 THE SEQUENTIAL INTERACTION OF THE T CELL ANTIGEN RECEPTOR WITH TWO DISTINCT CYTOPLASMIC TYROSINE KINASES, Arthur Weiss, Bryan A. Irving, Andrew C. Chan and Makio Iwashima, Howard Hughes Medical Institute, Depts. of Medicine and of Microbiology and Immunology, U.C.S.F., San Francisco.

Stimulation of the T cell antigen receptor (TCR) induces protein tyrosine phosphorylation. A 17 residue sequence motif, termed the antigen recognition activation motif (ARAM), contained in the TCR ζ and CD3 chains is responsible for coupling the receptor to cytoplasmic protein tyrosine kinases (PTKs). Two families of PTKs have been implicated, the Src and Syk/ZAP-70 families. Here, we provide genetic and biochemical evidence that, in the Jurkat T cell leukemic line, signal transduction is initiated by the Src-family PTK lck, when it phosphorylates two critical tyrosine residues within an ARAM. In turn, a second PTK, ZAP-70, is recruited to a doubly-phosphorylated ζ ARAM via both of its SH2 domains where it is phosphorylated by lck. Thus, the TCR uses ARAMs to interact with two distinct PTKs in a sequential and coordinated manner.

RAS/RHO/RAP

I 019 SIGNALING THROUGH RAS TO PROTEIN KINASES. Anne B. Vojtek and Jonathan A. Cooper, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104.

We have been searching for proteins that interact with the GTPase, Ras. Oncogenic activation of Ras stimulates the MAP kinase pathway, and dominant-negative mutants of Ras interfere with mitogen stimulation of MAP kinase. Several laboratories have obtained evidence that the protein kinase Raf is an intermediary between Ras and MAP kinase kinase. We have used the yeast 2-hybrid system of Fields and Song, modified by S. Hollenberg, to search for proteins that interact with mammalian Ras. Of over 14 million transformants screened, 21 gave positive signals with Ras and not with negative control plasmids. The 21 interacting plasmids fall into 8 sequence classes. Of these, 5 classes of sequences interact with wildtype Ras and not with effector domain mutants of Ras, suggesting that they may represent effectors of Ras. 2 of the 5 classes are derived from cRaf and A-Raf. *In vitro* biochemical studies confirm that the GTP form of Ras (but not the GDP form) binds tightly to the region of Raf contained in the clones. The 81-residue Ras-interaction domain of Raf is conserved through the Raf family. The c110 allele of *Drosophila raf* represents a partial loss-of-function mutation. This allele has a single residue substitution within the Ras-interaction domain. Making the same substitution in the Ras-interaction domain of cRaf blocks binding to Ras-GTP *in vitro*, consistent with a functional interaction between Ras-GTP and the Ras-interaction domain of the *Drosophila* Raf protein *in vivo*. Binding of Raf to Ras-GTP could bring Raf to the membrane, thereby leading to activation of Raf. The additional clones of candidate Ras-interacting proteins will be discussed.

I 020 THE BIOLOGICAL FUNCTION OF RHO AND RAC, Alan Hall[1], Anne J. Ridley[2], and Hugh Paterson[3], [1] MRC Laboratory for Molecular Cell Biology, University College London, Gower Street, London WC1E 6Bt, [2] Ludwig Institute for Cancer Research, 91 Riding House Street, London W1, [3] Chester Beatty Labs, Fulham Road, London SW3 6JB.

The rho subfamily of ras-related small GTPases comprises at least eight different proteins. We have been examining the biological role of two of these, rho and rac, in different cell types. We previously showed that in SWISS 3T3 cells, rho regulates a signal transduction pathway linking extracellular factors to the assembly of focal adhesions and actin stress fibres (1). Rac, on the other hand, links extracellular factors to the polymerization of actin at the plasma membrane to form lamellipodia and membrane ruffles (2). Rho and rac are ubiquitous proteins and we have also examined their role in other cell types. In the epithelial cell line, MDCK, we have shown that ras and rac, but not rho, are required for the early morphological responses observed after addition of scatter factor (also known as hepatocyte growth factor). Microinjection of recombinant ras and rac can mimic these early responses but they do not in themselves lead to a scattered phenotype; this requires additional step(s) including new gene transcrition.
We have cloned a ubiquitous 50KDa rhoGAP cDNA encoding a protein that stimulates the GTPase acrivity of at least three rho-like genes, rho, rac and CDC42. However, rhoGAP is only one of five GAPs so far identified for this subfamily of GTPases. We are currently looking at the role of these and other proteins in regulating the activity of rho-like GTPases.

1. Ridley A.J. and Hall A. (1992) Cell 70:389-399.
2. Ridley A.J., Paterson H.F., Johnson C.L., Diekmann D. and Hall A. (1992) Cell 70:401-410.

Mitogen Activation Protein Kinase Signalling

I 021 ORGANIZATION AND DYNAMICS OF MAP-KINASE ACTIVATION PATHWAYS, Beverly Errede[1], Beverly M. Yashar[1], Kenji Irie[2], and Kunihiro Matsumoto[2]. [1]University of North Carolina, Chapel Hill, NC, [2]Nagoya University, Nagoya, Japan.

In *S. cerevisiae,* MAP-kinase (MAPK) activation pathways mediate physiological responses to diverse signals. The best understood of these involves the response to mating pheromones. Pheromone-receptor binding informs cells of the proximity of a mating partner and induces differentiation to a mating competent state. Signal transmission is mediated by a G-protein that stimulates the sequential phosphorylation and activation of protein kinases which are conserved from yeast to mammals. In this pathway, Ste11 (*Ste=Sterile*), a MEK-kinase (MEKK), phosphorylates and activates Ste7, a MAPK/ERK-kinase (MEK). Ste7 in turn phosphorylates and activates the redundant MAPK related Fus3 and Kss1 enzymes. Another signaling pathway in yeast that uses a MAPK homolog is the growth response pathway. This pathway regulates cell wall construction, a process that is especially important at regions of directed growth during cell proliferation or mating differentiation. In this pathway the yeast protein kinase C isozyme, Pkc1, stimulates the phosphorylation cascade which consists of Bck1 (related to MEKK), the redundant Mkk1 and Mkk2 enzymes (related to MEK) and Mpk1 (related to MAPK).

We exploited these two well defined signal transduction pathways to identify mutations that cause aberrant MEK function. We identified the *MKK1-6* mutant allele by its ability to suppress the cell wall related growth defects of *pkc1* or *bck1* deletion mutants. The analogous amino acid substitution in Ste7 allowed the Ste7-6 derivative to activate the mating response pathway in the absence of the G_β signal transducer and also to substitute for Mkk1/Mkk2 in the growth response pathway. Thus, the basis for the hyperactivation phenotype may be applicable to the MEK family in general. To understand the biochemistry of this hyperactivation, we compared activity of the Ste7 and Ste7-6 enyzmes. The Ste7-6 enzyme is not constitutively active but still requires modification by Ste11 (or another MEKK) for its activity. Our current data are most consistent with the notion that Ste7-6 is a "Km variant" which favors activation by its own or other MEKKs.

I 022 DISSECTION OF THE TORSO SIGNAL TRANSDUCTION PATHWAY IN DROSOPHILA, Norbert Perrimon, Howard Hughes Medical Institute, Department of Genetics, Harvard Medical School, 200 Longwood Avenue, Boston, MA 02115

Cell fate choice at the anterior and posterior embryonic termini of the *Drosophila* embryo requires the activation of a signal transduction pathway that involves the receptor tyrosine kinase *torso*. When *torso*, which is uniformly distributed in the egg cell membrane becomes locally activated at the termini, it triggers a phosphorylation cascade that culminates with localized expression of the transcription factors *tailless* and *huckebein*. Several genes have been characterized that are involved in torso signaling: the GTP binding protein *ras-1*, the guanine nucleotide exchange factor *Son of sevenless* (*Sos*), the kinases *raf* and *MPKK*, and the transcription factors *tailless* and *huckebein*. Genetic and molecular evidence supports a model in which these proteins lie in the same biochemical pathway; i.e., when activated by its ligand the membrane bound receptor tyrosine kinase *torso* initiates a signal transduction pathway mediated through *Sos* and *ras-1* which in turn activates a phosphorylation cascade mediated by the kinases *raf* and *MPKK* which ultimately control the localized expression of the transcription factors *tailless* and *huckebein*. In addition, the *torso* signaling pathway involves *corkscrew*, a putative protein tyrosine phosphatase, with two SH2 domains, that acts as a positive transducer possibly by "up-regulating" the activity of *torso* or one of the other components of the terminal class pathway.

Small GTP Binding Protein Regulators

I 023 REGULATION AND FUNCTION OF RAS PROTEINS IN THE CONTROL OF CELLULAR GROWTH AND DIFFERENTIATION. Julian Downward, László Buday, Tanya Basu, Patricia Warne, Bengt Hallberg, Sydonia Rayter, Pablo Rodriguez Viciana, Sean Egan, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, U.K.

Ras proteins are key components of signal transduction pathways linking extracellular stimuli to the regulation of cellular growth and differentiation. They bind GTP and catalyse its hydrolysis to GDP, being biologically active when GTP-bound and inactive when GDP-bound. The activation state of Ras is controlled by the opposing influences of two different proteins families: the GTPase activating proteins (GAPs) and the guanine nucleotide exchange factors. In most cell types the activity of the guanine nucleotide exchange factor Sos is controlled through its association with receptors through the SH2/SH3 containing adaptor protein Grb2. Another important component of this pathway is the Shc protein which associates with Grb2 in activated cells, but does not bind Sos directly. The key role of Shc in the regulation of guanine nucleotide on Ras will be discussed.
Downstream signalling from Ras has recently been clarified with the observation that Ras interacts directly with the Raf family of serine/threonine kinases. This interaction can also been seen to be stimulated in whole cell extracts after stimulation of the cells (for example through the T cell receptor). The ability of Ras.GTP interaction with Raf to lead to activation of its kinase activity will be addressed. In addition, Ras can interact with other potential effector proteins in a GTP-dependent manner through its effector binding site. The ability of Ras to activate the MAP kinase pathway is well established, but the existence of other key signalling pathways downstream of Ras/Raf remains highly probable.

I 024 REGULATION OF RAS ACTIVATION, Larry A. Feig, Tufts University School of Medicine, Boston, MA, 02111

Activation of Ras proteins is a key step in the transmission of signals emanating from all receptors studied to date that act through membrane associated tyrosine kinases and some receptors that act through heterotrimeric G proteins. Ras proteins become activated by exchanging GTP for prebound GDP. The rate limiting step, the release of prebound GDP, is catalyzed by a family of proteins known a Guanine Nucleotide Exchange Factors (GEFs). Some GEFs (mSOS-1 and mSOS-2) are expressed ubiquitously, whereas some (Ras-GRF and Vav) have more limited tissue expression patterns. The latter therefor may play a role in tissue specific Ras function. Ras becomes inactivated by hydrolysis GTP to GDP. This process is catalyzed by GTPase Activating Proteins (GAPs).

We have focussed our attention on Ras-GRF, which is expressed preferentially in brain and have been comparing its properties to the ubiquitously expressed SOS proteins. By using in situ hybridization, we have detected a distinct pattern of GRF expression in brain that is developmentally regulated. Interestingly, this pattern is different from that of SOS.

SOS couples to tyrosine kinase receptors by its ability to bind the SH3 domains of the adaptor protein, Grb2. The SH2 domains of Grb2, in turn, bind tyrosine phosphorylated residues on activated receptors, such as that for EGF. We have therefore studied GRF interactions with this system and found that unlike, SOS, GRF does not bind Grb2 in vitro or in vivo. Moreover, unlike SOS, GRF cannot be detected in a complex with the EGF receptor upon growth factor addition to cells. These results suggest Ras-GRF is coupled to another signalling system that is used in brain to activate Ras. Experiments will be discussed whose aim is to detect this novel input pathway.

I 025 REGULATOR AND TARGET PROTEINS FOR *Ras* AND *Rab*3A, Yoshimi Takai[1,2], Kozo Kaibuchi[1,2], Takuya Sasaki[1], Hiromichi Shirataki[2], Kazuma Tanaka[1], and Hiroyuki Nakanishi[1], [1]Department of Biochemistry, Kobe University School of Medicine, Kobe 650 and [2]Department of Cell Physiology, Institute for Physiological Sciences, Okazaki 444, Japan.

Three GDP/GTP exchange proteins have been identified for mammalian *Ras*, m*Cdc*25, m*Sos*, and *Smg* GDS. Recent extensive studies have clarified that m*Sos* receives a signal from the tyrosine kinase-type membrane receptors through Grb2 and converts GDP-*Ras* to GTP-*Ras* on the plasma membrane. We have investigated detailed kinetic properties of *Smg* GDS, which has been discovered in our laboratory, in comparison with those of m*Sos* and m*Cdc*25 with Ki-*Ras* as a common substrate. We have found that *Smg* GDS has a potential activity to translocate membrane-bound GTP-Ki-*Ras* to the cytosol after it is converted from GDP-Ki-*Ras* by the action of m*Sos* or m*Cdc*25. Consistently, we have identified a direct target protein for *Ras*, which activates MAP kinase kinase (MEK) in a cell-free system, in the cytosol of *Xenopus* oocyte. We have highly purified this protein factor and named it REKS (*Ras*-dependent ERK Kinase Stimulator). REKS is apparently different from *Raf* or *Mos*, which has been reported to activate MEK.

The *Rab* family is involved in intracellular vesicle transport. The mode of action of *Rab* in vesicle transport is proposed as follows: GDP-*Rab* staying in the cytosol is first converted to GTP-*Rab*. GTP-*Rab* then interacts with its specific target protein on the vesicle, eventually transporting and docking the vesicle to a specific acceptor membrane. After the fusion of the vesicle with the membrane, GTP-*Rab* is converted to GDP-*Rab* which is translocated to the cytosol. We have discovered a regulatory protein which may regulate this cyclical translocation of *Rab* between the membrane and the cytosol, and named it *Rab* GDI (GDP Dissociation Inhibitor). We have recently discovered a target protein for *Rab*3A, which is particularly implicated in neurotransmitter release, and named it Rabphilin-3A. Rabphilin-3A is composed of two functionally different domains: N-terminal *Rab*3A-binding and C-terminal Ca^{2+}- and phosphatidylserine-binding domains. Rabphilin-3A also shows a *Rab*3A GTPase Activating Protein-inhibiting activity. Rabphilin-3A may keep *Rab*3A in the GTP-bound form during its action as a target molecule for *Rab*3A. Rabphilin-3A as well as *Rab*3A appears to play a crucial role in neurotransmitter release. In this symposium, we will describe the function and mode of action of these regulatory and target proteins for *Ras* and *Rab*3A.

Heterotrimeric G Proteins

I 026 G PROTEIN SUBUNIT INTERFACES, Eva J. Neer, Carl Schmidt, Denise Spring, Thomas C. Thomas, and Fei Yi, Cardiovascular Division, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115.

Heterotrimeric GTP-binding proteins, made up of α and $\beta\gamma$ subunits, transmit signals from a variety of receptors to intracellular effectors. The association and dissociation of the α and $\beta\gamma$ subunits plays a central role in the mechanism of action of this class of G proteins. Information about which surfaces of the α and $\beta\gamma$ subunits face each other can be obtained by introducing chemical crosslinks. We have previously shown that 1,6-bismaleimidohexane (BMH), which crosslinks cysteine residues, can crosslink several α subunits to $\beta\gamma$ (Yi F, Denker BD, and Neer EJ [1991] J Biol Chem 266:3900). Mutagenesis of the cysteines in α_o showed that changing Cys215 to alanine prevented the crosslinking, but did not affect the ability of the mutated α subunits to interact normally with $\beta\gamma$. Cys215 is located in one of the most conserved regions of the α subunits, suggesting that it is in a region critical for the functions of all α subunits. It is near a region that changes conformation with the type of guanine nucleotide bound. This conformational change is likely to be important in regulating the affinity of α_o for $\beta\gamma$. The surface of α to which $\beta\gamma$ may bind overlaps the putative effector binding region. If the surfaces do overlap, then it is unlikely that α could bind effector and $\beta\gamma$ simultaneously. Similar mutational analysis is underway to define the crosslinking site on the β subunit.

We have also investigated the site on the γ subunit that defines its interaction with the β subunit. Not all combinations of β and γ subtypes can form dimers: the γ2 subunit can form dimers with β1 and β2, but the γ1 forms dimers only with β1. Analysis of a series of γ1/γ2 chimeras shows that when amino acids 36-49 of γ1 replaced amino acids 33-46 of γ2, the chimeric γ subunit could only dimerize with β1; the reciprocal chimera in which 14 residues from γ2 replaced the equivalent region of γ1 could form dimers with both β1 and β2. We conclude that this 14 amino acid region is sufficient for γ1 to discriminate between the β subunits. Identifying the subunit contact surfaces is a start toward defining the molecular basis for the specificity of G protein subunit interactions. (Supported by grants from the NIH and American Heart Association.)

I 027 CRYSTALLOGRAPHIC ANALYSIS OF GTP AND GTP BOUND FORMS OF THE Giα SUBUNIT, S. Sprang[1,3], D. Coleman[1], E. Lee[2], A. Berghuis[1], M. Mixon[3], M. Linder[2], A. Gilman[2], [1]Howard Hughes Medical Institute, [3]Department of Biochemistry, and [2]Department of Pharmacology, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd. Dallas, TX 75235-9050.

The three-dimensional structure of Giα complexed with the non-hydrolyzable substrate analog guanosine-5'-0'-3-thiotriphosphate (GTPγS), is being determined by X-ray crystallography. Gi, a member of the heterotrimeric G-protein family, downregulates adenylyl cyclase and opens potassium channels through its interaction with the cytoplasmic domain of the somatostatin and related receptors. Signal transduction is mediated by receptor induced exchange of GDP for GTP, and by hydrolysis of bound GTP to GDP by the intrinsic GTPase activity of the alpha subunit. Crystals of the Giα•GTPγS (space group $P3_{1/2}21$ a=80.5Å, c=106.3Å) have been obtained that diffract beyond 2.0Å resolution, and a complete 2.0Å data set has been measured at the CHESS F1 beamline. Phase determination is in progress by a combination of heavy atom isomorphous replacement and multiwavelength anomalous dispersion using data collected at the X4a beamline at NSLS from isomorphous crystals of selenomethionyl-Giα•GTPγS. Crystals of the GDP-bound form of Giα have been obtained in a different crystal form (space group $I4_{1/3}$, a=121.3Å, c=67.7Å) and diffract to 3.0Å resolution. A "conformational mutant" of Giα (G203A) binds and hydrolyzes GTP and activates adenylyl cyclase, but fails to undergo the conformational change required to release $\beta\gamma$ subunits, has also been crystallized in both GTPγS (space group $P4_{3/1}2_12$, a=77.1Å, c=144.6Å, diffraction to 3.0Å) and GDP bound forms. The latter crystals resemble those of the "wild type" GDB bound crystals. The structure of this mutant should provide insight into the mechanism of the GTP-induced conformational changes. Additionally, constitutively active muteins of Giα (R178C, Q204L) which bind but do not hydrolyze GTP have been crystallized as complexes with GTPγS.

Transcription

I 028 HORMONAL CONTROL OF TRANSCRIPTION: THE cAMP-RESPONSIVE PATHWAY, Marc Montminy, Paul Brindle, Robert Armstrong, Kevin Ferreri, and Jonathan Arias, The Salk Institute, La Jolla, CA 92037.

Many growth factors and hormones regulate cellular activity through second messengers which correspondingly induce multifunctional protein kinases. The second messenger cAMP, for example, regulates a number of eukaryotic genes by mediating the protein kinase A (PKA) dependent phosphorylation of the nuclear factor CREB at Ser133. Although phosphorylation may regulate transcription factors at a number of levels including DNA binding and nuclear translocation, CREB belongs to a class of activators whose trans-activation potential is specifically enhanced. In this regard, CREB contains two functionally distinct activation domains--a constitutive glutamine rich domain termed Q2, and a kinase inducible domain, termed KID--which act synergistically to stimulate transcription in response to cAMP[1]. KID activity alone is not sufficient to sustain a transcriptional response as illustrated by the CREM family of repressors, which contain KID domains but lack the Q2 region. In reconstituted systems, Q2 appears to stimulate transcription by interacting specifically with at least one component of the TFIID complex (dTAF$_{II}$110). As the transcriptionally inactive CREMα and β proteins lack sequences in Q2 which are necessary for binding dTAF$_{II}$110, our results suggest that these proteins may repress transcription because they are unable to interact with the basal transcription complex.

1. Brindle P., Linke S., Montminy M. (1993). Analysis of a PK-A Dependent Activator in CREB Reveals a New Role For the CREM Family of Repressors. Nature In press

Programmed Rearrangements and Active Cell Death

I 029 DOWN REGULATION OF WT p53 ACTIVITY INTERFERES WITH APOPTOSIS OF IL3-DEPENDENT HEMATOPOIETIC CELLS FOLLOWING IL3 WITHDRAWAL. Eyal Gottlieb[1], Rebecca Haffner[1], Thomas von Rüden[2], Erwin F. Wagner[2], and Moshe Oren[1], [1]Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel, and [2]Research Institute of Molecular Pathology (IMP), A-1030, Vienna, Austria.

Overexpression of wild-type p53 (wt p53) in wt p53-deficient leukemic cells induces apoptosis, which can be inhibited by hematopoietic survival factors. This suggests that p53 may contribute to survival factor dependence. To assess the role of wild-type p53 in mediating apoptosis following survival factor withdrawal, we interfered with endogenous p53 activity in IL3-dependent cells. Extended survival without IL3 was conferred by recombinant retroviruses encoding either a full-length p53 mutant or a C-terminal p53 miniprotein, both of which can act as negative-dominant inhibitors of wild-type p53. A similar partial protection was also conferred by a plasmid encoding p53 antisense RNA. In parallel, cells expressing negative-dominant p53 exhibited a greatly increased resistance to apoptosis induced by ionizing radiation. On the other hand, excess wild-type p53 activity failed to elicit apoptosis as long as IL3 was present. We propose that p53 is a positive, though not exclusive, mediator of survival factor dependence in hematopoietic cells. Thus, in addition to its demonstrated role in allowing the apoptotic death of irradiated cells, p53 appears to participate in central physiological pathways which are controlled by the availability of certain survival factors. Abrogation of wt p53 function may therefore contribute to cancer by allowing illegitimate survival under conditions which are otherwise prohibitory to cell survival.

Signalling Down-Regulation

I 030 REGULATION OF G PROTEIN-COUPLED RECEPTORS, Jeffrey L. Benovic, Priya Kunapuli, Jorge Gomez, Chong M. Kim, and Guanghui Kong, Department of Pharmacology, Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Many transmembrane signalling systems consist of specific G protein-coupled receptors that transduce the binding of extracellular ligands (hormones, neurotransmitters, odorants, light, etc.) into intracellular signalling events. G protein-coupled receptors modulate the activity of a wide variety of effector molecules including adenylyl cyclase, cGMP phosphodiesterase, phospholipase C, phospholipase A2, and various ion channels. Two of the best characterized G protein-coupled receptors are the β_2-adrenergic receptor (β_2AR), which mediates catecholamine stimulation of adenylyl cyclase, and the visual "light receptor" rhodopsin, which mediates phototransduction in retinal rod cells. In both of these systems a rapid loss of responsiveness occurs following receptor activation. This rapid activation-dependent loss of receptor responsiveness appears to be mediated by specific G protein-coupled receptor kinases (GRK) that have the unique ability to recognize and phosphorylate their receptor substrates only when they are in their active conformations. The β-adrenergic receptor kinase (βARK) and rhodopsin kinase have been implicated as the major kinases involved in the stimulus-dependent phosphorylation of the β_2AR and rhodopsin, respectively. The subsequent uncoupling of the receptor and G protein is then mediated by arrestin proteins that specifically bind to the phosphorylated and activated form of the receptor.

A growing body of evidence suggests that βARK and rhodopsin kinase are members of a multigene family. While structural information on the GRK family was initially provided by the isolation of a cDNA encoding bovine βARK, additional cloned members of this family include βARK2, rhodopsin kinase, IT11, GRK5 and GRK6 as well as two *Drosophila* kinases GPRK-1 and GPRK-2. A comparison of the amino acid sequences of the GRKs suggests that there are two major branches of the GRK family tree. βARK2 and *Drosophila* GPRK-1 appear to be the most similar to βARK with amino acid identities of 84% and 64%, respectively. In contrast, rhodopsin kinase, IT11, GRK5, GRK6 and *Drosophila* GPRK-2 have significantly lower homology with βARK (35-40% amino acid identity) and form a separate branch of the tree. The phylogenetic classification of the GRK family is further supported by functional analyses of the various GRKs. *In vitro* studies have demonstrated that βARK and βARK2 share a very similar substrate specificity both at the level of amino acid preference (they phosphorylate serine-containing peptides with N-terminal acidic residues), receptor phosphorylation (β_2AR, m2 muscarinic cholinergic and substance P receptors are good substrates *in vitro*), and potential mechanism of cellular activation (interaction with G protein $\beta\gamma$ subunits). βARK, βARK2, and *Drosophila* GPRK-1 also appear to be ubiquitous proteins being expressed in a variety of tissues. While at present little is known about the function of GRK6, GRK5, IT11 and *Drosophila* GPRK-2, the similarities between these four proteins suggests that they may well share common roles in the cell. Agonist-promoted receptor phosphorylation by specific G protein-coupled receptor kinases may well serve as a general mechanism for regulating a wide variety of receptors in diverse organisms.

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I 031 NEW PROTEINS IN THE PHOTOTRANSDUCTION CASCADE. Krzysztof Palczewski[1,2], Wojciech Gorczyca[1], Hiroshi Oghuro[1], Mark P. Gray-Keller[3], and Peter B. Detwiler[3], Departments of [1]Ophthalmology, [2]Pharmacology, and [3]Physiology and Biophysics, University of Washington School of Medicine, Seattle, Washington, 98195.

In retinal rods, photolysis of rhodopsin triggers of cascade of enzymatic reactions that increases cGMP hydrolysis and generates an electrical signal by causing closure of cGMP-gated ion channels in the photoreceptor outer segment. This leads to a decrease in internal Ca which activates guanylate cyclase and promotes photoresponse recovery by stimulating the resynthesis of cGMP. It was believed that the inactivation of photolyzed rhodopsin requires phosphorylation of the receptor, binding arrestin, and reduction of the chromophore all-*trans*-retinal to all-*trans*-retinol. We also found that another protein, p^{44}, is involved in the quenching process. p^{44}, which is a shorter splice variant of arrestin, binds avidly to bleached or bleached and phosphorylated rhodopsin. The complex is stable as long as the retinal is not reduced to retinol by the dehydrogenase. In another step of phototransduction, we found that the activation of guanylate cyclase by low Ca is mediated by ~ 20 kDa protein purified from bovine rod outer segments using DEAE-Sepharose, hydroxylapatite and reverse-phase chromatographies. In a reconstituted system, this protein restores the Ca-sensitive regulation of guanylate cyclase, and when dialyzed into functionally-intact lizard ROS, decreases the sensitivity, time to peak and recovery of the flash response. [Supported by NIH Grants EY09449]

I 032 G PROTEIN MEDIATED ADAPTATION—A NOVEL SIGNALING PATHWAY IN YEAST, David E. Stone[1], Holly F. Stratton[1], and Steven I. Reed[2], [1]Department of Biological Sciences, University of Illinois, Chicago, IL 60607, [2]The Scripps Research Institute, San Diego.

The mating signal transduction pathway of the budding yeast *S. cerevisisae* is mediated by a receptor-coupled heterotrimeric G protein. Upon receptor activation, the pheromone responsive G_α protein, Gpa1, releases the $G_{\beta\gamma}$ subunit, which then interacts with an unknown effector and propagates the signal to mate. The consequences of the mating signal run the gamut of cellular responses: cell cycle arrest, regulation of gene expression, cytoskeletal reorganization, polarized growth, cell-cell interaction, and membrane fusion. Many groups are attempting to understand how the mating signal is transduced. Our goal is to understand how it is turned off. Genetic evidence suggests that in addition to negatively regulating the pheromone response by sequestering $G_{\beta\gamma}$, Gpa1 interacts with an effector and, like other G_α proteins, transduces a signal. The Gpa1-mediated signal is an adaptive one. It inhibits the mating response and allows cells to resume cell division in the continued presence of agonist. Recently, we have made an observation concerning the timing of this adaptive signal. When an asynchronous culture *constitutively* expressing a hyperadaptive form of Gpa1 (E364K) is treated with a dose of pheromone that permanently arrests cell cycle progression of the wildtype strain, the cells do not behave in a uniform manner. Eighty percent of the E364K cells respond to pheromone and then recover; twenty percent appear to be insensitive. However, when cells constitutively expressing E364K are synchronized in early G_1 by centrifugal elutriation and then treated with pheromone, we see a 100% response followed by recovery. This observation is important for two reasons. First, it argues strongly in favor of Gpa1-mediated adaptation, and against the idea that E364K allows growth in otherwise lethal concentrations of pheromone by conferring insensitivity. Second, it suggests the existence of a lag between the induction of the mating signal and the mechanism that promotes recovery. Our reasoning is as follows: In an asynchronous culture, cells that have just passed START (the point in the cell cycle where progression is blocked by pheromone) may have sufficient time to induce the adaptive signal before reaching START again. Thus, they appear insensitive. Cells in early G_1, on the other hand, do not have time to fully induce the adaptive mechanism before reaching the arrest point. This interpretation suggests, in turn, that pheromone is necessary to fully induce the adaptive signal, even in cells expressing a hyperadaptive form of Gpa1. In addition, our data indicate that at least one of the targets of the Gpa1-induced adaptive signal is the pheromone responsive G_β subunit, Ste4. Using a strain of yeast whose viability depends on mutational inactivation of Gpa1-mediated adaptation, we have isolated a large number of adaptation$^-$ mutants, which we are in the process of characterizing genetically. Thus far, we have identified dominant mutations in three linkage groups that disrupt the ability of Gpa1 to stimulate adaptation. One of these linkage groups is *STE4*; novel alleles of *STE4* that act as dominant negatives in relation to hyperadaptive alleles of *GPA1* will be discussed. Our screen also yielded six recessive adaptation$^-$ mutants. These appear to fall into one complementation group.

G Protein Effectors

I 033 G PROTEIN REGULATION OF Na-H EXCHANGE, Tatyana Voyno-Yasenetskaya, Robin L. Gilbert, Bruce Conklin, Henry R. Bourne, and Diane L. Barber, University of California, San Francisco, CA 94143.

Activity of the ubiquitously expressed Na-H exchanger, NHE1, plays an integral role in cell growth, differentiation, and secretion. Although a number of hormone and neurotransmitter receptors coupled to heterotrimeric G proteins regulate NHE1 activity, the specific G proteins involved in this regulation remain to be identified. We recently reported that the β-adrenergic receptor (βAR) and other receptors that stimulate adenylylcyclase by activating G_s, stimulate the exchanger by a guanine nucleotide-dependent mechanism that is independent of receptor coupling to G_s. We have now used a mutational analysis of several structural domains shared by GTPases to determine that two recently identified Gα subunits of unknown function, α12 and α13, regulate the exchanger. Point mutations at conserved codons in α subunits inhibit intrinsic GTPase activity, inducing constitutive activation in an agonist-independent fashion. Expressing mutationally activated α subunits in HEK293 cells revealed that activated α13, but not wild type α13, increased NHE1 activity. Mutationally active α12 had no effect on control NHE1 activity, however, it inhibited the stimulatory action of activated α13. Because the receptors coupled to α13 had not been identified, agonist-induced activation of α13-stimulated NHE1 activity was studied using a chimeric $\alpha 13/\alpha_z$ protein. The C-terminus of several α subunits confers the specificity of receptor recognition, and transfering this region to a different α subunit confers the same specificity. We therefore used the D_2-dopamine receptor, which activates α_z, to stimulate an $\alpha 13/\alpha_z$ chimera in which the C-terminus of α13 was replaced by cognate residues of α_z. D_2 agoinsts stimulated a rapid increase in NHE1 activity in cells expressing the D_2R and the $\alpha 13/\alpha_z$ chimera, but had no effect on control NHE1 activity in cells expressing either the receptor alone or the receptor plus wild type α13. The role of α13 in mediating βAR activation of NHE1 was studied by inhibiting α subunit expression with antisense oligonucleotides. In preliminary studies, antisense oligonucleotides directed against α13 attenuated isoproterenol-stimulated exchange activity, but had no effect on agonist-induced cAMP accumulation. In contrast, oligomers antisense to α_s attenuated isoproterenol-stimulated cAMP synthesis but had no effect on agonist activation of NHE1. These results suggest that Gα13 may constitute a new and divergent signalling pathway for the βAR. Additionally, the functional coupling of Gα13 to NHE1 indicates that ion exchangers may represent a class of G protein-coupled effectors. Because both Gα13 and NHE1 are ubiquitously expressed, this signalling pathway may be significant in the function of all cell types. Finally, the techniques we applied to identify the action of Gα13 included using mutationally actived Gα subunits as well as chimeric α subunits designed to respond to receptor-specific activation. These approaches may be useful in characterizing the regulation and function of other GTPases.

Protein Phosphatases

1034 PROTEIN TYROSINE PHOSPHATASES: SIGNAL TRANSDUCTION AND CATALYTIC MECHANISMS, Jack E. Dixon, Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, Michigan

The diversity of the protein tyrosine phosphatases (PTPases) is rather astonishing and underscores the central role these catalyst plays in regulating cellular function. Although more phosphatases are likely to be discovered, there are several emerging themes which are common to PTPase regulation and catalysis. The family of intracellular PTPases appears to consist of a catalytic domain and other domains. One of the functions of the other domain appears to be to direct the PTPase to specific intracellular locations. Several examples of this PTPase targeting will be discussed. It also appears that most, if not all of the PTPase will utilize a common mechanism for catalysis. The catalytic residues important in formation of a thiol-phosphate intermediate will be discussed. In addition, specific Arg, Asp and Glu residues which function in substrate binding as well as in general acid and general base catalysis will be reported. This work was supported in part by grants from the National Institutes of Health and the Walther Cancer Institute.

1035 PHOSPHORYLATION OF CDC25 IN FISSION YEAST, Michel Charbonneau, Guy Lenaers, Robert Kovelman and Paul Russell, The Scripps Research Institute, La Jolla.

Cdc2/cyclin B, the kinase that is responsible for bringing about mitosis, is maintained in an inhibited state prior to mitosis due to the phosphorylation of Cdc2 on Thr14 and Tyr15. The Tyr15 phosphorylation is carried out by Wee1 protein kinase, while the dephosphorylation of both sites is performed by Cdc25 protein phosphatase. It has been proposed that Cdc2/cyclin B might also stimulate Cdc25 by direct phosphorylation, thus completing a positive feedback loop that is perhaps important for the induction of mitosis. Studies from several groups have shown that Cdc25 becomes hyperphosphorylated when *Xenopus* eggs are arrested in M-phase. This hyperphosphorylated form of Cdc25 appears to be ~2-5 fold more active when assayed *in vitro*. These studies suggest that phosphorylation of Cdc25 might contribute to the induction of M-phase in *Xenopus* eggs. We have carried out a series of studies aimed at evaluating the role of Cdc25 phosphorylation in the fission yeast *Schizosaccharomyces pombe*. These studies support the conclusion that the mitotic induction activity of Cdc25 is stimulated by phosphorylation, and show that many of these phosphorylations can be carried out by Cdc2/cyclin B kinase *in vitro*. However, our *in vivo* studies also show that these phosphorylations occur prior to the activation of Cdc2/cyclin B kinase that occurs at the transition from G_2 to M-phase. The significance of these finding will be discussed.

Kinase Cascade

1036 REGULATION OF THE MAP KINASE/ERK CASCADE, Shui-Chan Xu, David Robbins, Michele Hutchison, Erzhen Zhen, Jessie Hepler, Mangeng Cheng, and Melanie H. Cobb. Department of Pharmacology, U.T. Southwestern Medical Center, Dallas, TX 75235

The MAP kinase/ERK cascade is activated by numerous cellular stimuli and is believed to be important in the control of proliferation and differentiation. We are currently investigating the upstream regulation, specificity, and structures of ERKs, MEKs, and MEKK to understand how they contribute to distinct cellular responses. There is evidence to suggest that there are multiple isoforms of each of these protein kinases. For example, we find that PC12 cells and animal tissues contain at least three different MEK activities as determined by chromatographic behavior and substrate specificity. Two of these phosphorylate the protein kinase ERK3 on serine 189. In addition, at least two of these are phosphorylated and activated by the same upstream kinases, Raf and MEKK, in vitro. A MEKK clone expressed in 293 cells is phosphorylated primarily on serine residues and is inactivated by 50% or more upon dephosphorylation with phosphatase 2a in vitro. A C-terminal fragment encoding only the catalytic domain of MEKK is also phosphorylated in 293 cells and inactivated with phosphatase 2a. A more detailed understanding of this pathway may help to account for the complexity of actions attributed to it.

I 037 cAMP-DEPENDENT PROTEIN KINASE: TEMPLATE FOR FOLDING, CATALYSIS, POSTTRANSLATIONAL ORGANIZATION, AND INHIBITOR BINDING, Susan S. Taylor, Friedrich W. Herberg, Joseph A. Adams, Igor Tsigelny, Jianhua Zheng, Wei Wen, and Robin M. Gibson, University of California San Diego, Department of Chemistry, 9500 Gilman Drive, La Jolla, CA 92093-0654.

Protein kinases, critical for signal transduction, must not only be accurate catalysts, they must also respond precisely to on and off signals. Although cAMP-dependent protein kinase (cAPK) is one of the simplest members of the protein family, it nevertheless provides a template for the folding of the polypeptide chain and localizes the numerous conserved residues at the active site that contribute to ATP binding and catalysis. These features will, in general, be conserved throughout the family. However, the crystal structure also provides an example of how posttranslational modifications contribute to overall conformance stability and function. For example, an essential phosphorylation site is found at the active site of many protein kinases. In the catalytic (C) subunit, this is Thr197, and in the active conformation of the C-subunit this stable phosphate is anchored by interactions with several side chains including R165, K189, and H87. Mutation of Thr197 to Asp not only influences catalysis, it also interferes with binding of the regulatory (R) subunit. The R-subunits are competitive inhibitors of substrate peptides and bind to C with high affinity in the absence of cAMP. Inhibition by R requires a common consensus site that resembles a peptide substrate, but in addition, tight binding of R requires a peripheral site that flanks the surface of C surrounding Thr197. The other tight binding physiological inhibitor of C, the heat stable protein kinase inhibitor, PKI, is not sensitive to perturbations around Thr197. It also binds to the common consensus site, but high affinity binding requires a hydrophobic surface that is N-terminal to the consensus site. Perturbations in this region, such as R133A, render the C-subunit insensitive to PKI inhibition but still sensitive to inhibition by R. The C-subunit, therefore, can use different surfaces for establishing protein:protein interactions, and phosphorylation can selectively influence those interactions.

The C-subunit is also myristylated. The myristylation site lies at the N-terminus outside the conserved catalytic core with the acyl group anchoring the N-terminus to a hydrophobic pocket on the surface of the large lobe. The myristylation motif (residues 1-14) is linked to the catalytic core by a long A-helix that spans the surface of both lobes. An A-helix that precedes the conserved catalytic core is very likely a conserved feature of most protein kinases. While in the C-subunit, this A-helix links the core to the acylation motif, in the tyrosine kinases, such as src, a similar A-helix probably serves as a linker between the core and the SH2 domain, thus orienting both the SH2 domain and the SH3 domain. The structure of the C-subunit thus can provide a model for organizing the domain structure of other protein kinases and these interactions of the kinase core with other proteins and/or structural motifs will be at least as important for overall function as the active site where catalysis takes place.

Cell Cycle

I 038 FOUR DISTINCT MODES OF CELL CYCLE CONTROL DURING EMBRYOGENESIS, Patrick H. O'Farrell, Frank Sprenger, and Bruce A. Edgar, Dept. of Biochem. and Biophys., UCSF, San Francisco, CA. 94143-0448

The discovery that key cell cycle regulators are conserved among all eukaryotes led to the suggestion that there is a universal cell cycle. **This suggestion is inaccurate in that there are many types of cell cycles**. The conserved cell cycle regulators are designed to accept numerous regulatory inputs, and together these regulators constitute a flexible regulatory machine that runs a variety of cell cycles differing in their fundamental characteristics. We have defined transitions in the mechanisms governing cell cycles during development of Drosophila, and have shown that cell cycle progression is tightly coordinated with gene expression and developmental events.

In Drosophila embryos cell cycles 2 through 9 are exceedingly fast (8.4 min) nuclear division cycles within a syncytial cytoplasm. The cycle slows gradually during the next four divisions, and then changes abruptly at the mid-blastula transition. The cell cycle acquires a G2 phase at the mid-blastula transition, and the length of the subsequent cycle (averages about 1.5 hr) is determined by the timing of progression from G2 to mitosis. This timing is not controlled by levels of cyclins because the levels of these regulators exceed the amount needed. Rather the timing of progression from G2 to M is determined by the timing of expression of the *string* phosphatase. String removes inhibitory phosphates from the cdc2 kinase. The resulting activation of the cdc2 kinase drives the cycle. The spatial patterning of the post mid-blastula transition cell cycles is dictated by transcriptional controls the direct expression of the *string* gene in a pattern of precisely timed pulses.

Prior to the mid-blastula transition constitutively high levels of the *string* phosphatase are provided maternally. Consequently, during the first 13 cycles there is no inhibitory phosphorylation of cdc2, and no G2. To study these rapid and synchronous early cycles we examined single embryos that were precisely staged. The level and state of phosphorylation of cyclins A and B, the cdc2 kinase, and String were determined and the activity of the cdc2 kinase measured.

Surprisingly, there is no oscillation in levels, phosphorylation or activity of cell cycle regulators during the first 7 cycles. Oscillations of several parameters (destruction of cyclins, phosphorylation on T_{161} of cdc2, and cdc2 kinase activity at mitosis) begin in cycle 8 and increase in magnitude in subsequent cycles. Genetic reduction of the maternal contribution of cyclin RNA's results in lengthening of cycles 10-13. This is the first in vivo demonstration that cyclin levels control the length of a cycle, however, it should be noted the cyclin levels do not control the length of the earlier cycles or the later cycles.

Thus, there are three types of cell cycles. Early cycles in which no oscillations are apparent (we believe these are driven by local oscillations controlled by the mitotic apparatus itself), transition cycles whose gradually increasing length is controlled by titration of cyclin synthetic capacity (the lengthening of these cycles is important to the timing of the mid-blastula transition), and the post mid-blastula transition cycles that are controlled by periodic transcription of the String phosphatase.

Poster Session I

I 100 SUBSTANCE K RECEPTOR DESENSITIZATION IS REGULATED VIA PKC. Jacqueline Alblas, Ingrid van Etten, Azra Khanum and Wouter H. Moolenaar. Dept. Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, The Netherlands.

Substance K (SK) is one of the mammalian tachykinins: neuropeptides which share a wide spectrum of biological effects including actions on sensory signaling, inflammation and cell proliferation. The receptor for SK belongs to the superfamily of G protein-coupled receptors and communicates to phosphoinositide-specific phospholipase C via a pertussis toxin-insensitive G protein. The SK receptor is capable of stimulating DNA synthesis in its target cells but only in concert with tyrosine kinase receptors. Like many G protein-coupled receptors, the SK receptor is subject to desensitization. Using an immunoprecipitating antibody, we found that the phosphorylation state of the receptor could be increased by stimulation of cells with SK or phorbol ester, indicating that PKC might play a role in regulating the SK receptor. Furthermore, TPA could completely inhibit the SK-induced PLC response, whereas inhibition of PKC resulted in a further enhancement of PLC activation. To investigate the regulation of the receptor by phosphorylation, we constructed a number of deletion mutants lacking most of the potential phosphorylation sites in the C-terminus. Two mutant receptors, deleted at aa 328 and 338, were stably transfected into RAT-1 cells. They showed ^{125}I-SK binding and G protein coupling indistinguishable from the wt SKR. Stimulation of these cells with SK gave rise to high responses of PLC and AC, which were no longer sensitive to TPA treatment or inhibition of PKC.

I 101 HYDROLYSIS OF PHOSPHATIDYLCHOLINE INDUCES HIV REPLICATION AND ACTIVATES NF-KB THROUGH A PKCζ-DEPENDENT PATHWAY. *F. Arenzana*, M.T. Diaz, B. Fernandez,*, I.Dominguez, J.L.Virelizier,* and J. Moscat. *Institut Pasteur, Paris, France.CBM-CSIC, Univ. Autonoma, Madrid, Spain.*

Activation of NF-KB transcription factor in monocytes and lymphocytes can be obtained by stimulation with physiological inducers like TNF, IL1 or , in the case of T lymphocytes, following engagement of the CD3-TCR complex. Additionally, expression of activated p21ras in human cells leads to transactivation of KB-dependent vectors. A characteristic shared by the above cited inducers of NF-KB is their capacity to induce hydrolysis of phosphatidylcholine (PC) by the phospholipase C (PLC). That suggests that, in human monocytes and T lymphocytes, the two types of target cells of HIV infection, phosphodiesterase-mediated hydrolysis of PC has the potential to be a major activation pathway leading to induction of nuclear NF-KB and transactivation of the HIV-enhancer.Using cell systems relevant to HIV pathogenesis, including an IL2-dependent T-cell clone, we demonstrate that specific hydrolysis of PC but not phosphatidylinositol, induces nuclear translocation of bona fide NF-KB and increases HIV enhancer activity and viral replication independently of classical, PMA-inducible, PKC, in chronically infected cells treated with exogenously added PC-PLC from Bacillus cereus. Furthermore, expression of a Bacillus cereus-PC-PLC gene induces an intense transactivation effect in co-transfected KB-dependent reporter vectors. Additionally, we demonstrate that overexpression in fibroblasts, of a PKCζ isoform, non-inducible by PMA, induces expression of elevated amounts of nuclear NF-KB and transactivates reporter vectors drived by KB enhancers. Finally, we prove that the transactivatory effect induced either by PC-PLC or TNF on KB-directed reporter vectors can be blocked by the expression of a transdominant negative mutated form of PKCζ carrying a K275W mutation in its catalytic domain.

I 102 IL-8 RECEPTORS: DETERMINATION OF THE DOMAINS WHICH ARE RESPONSIBLE FOR SIGNAL TRANSDUCTION, Ben-Baruch A. Johnston J.A. Oppenheim J.J. Kelvin D.J., Laboratory of Molecular Immunoregulation, Biological Response Modifiers Program, National Cancer Institute-FCRDC, Frederick, MD 21702.

IL-8 is a chemotactic cytokine (chemokine) and is known to be one of the major mediators of the inflammatory response. Two types of receptors for IL-8 - IL-8 receptors α and ß - have been cloned and sequenced. Both types of receptors are members of the Rhodopsin superfamily of transmembrane receptors. Although the two IL-8 receptors were shown to couple to G proteins and to activate PLC in neutrophils, it is still not known which segments of these receptors mediate these processes. The aim of this study is to determine which domains of IL-8 receptors couple to G proteins and confer upon the cells the responsiveness to IL-8.

In order to study this question we have constructed a number of substitution and deletion mutant IL-8Rα and IL-8Rß receptor genes. Mutations are clustered in the third intracellular loop as well as the carboxyterminus of both receptors. These two regions have been shown by others to be important in coupling G proteins and transducing signals by several such transmembrane receptors (i.e. adrenergic receptors). The effect of these mutations is being examined by transducing mutant receptor genes into 293 human embryonic kidney cells and monitoring IL-8 stimulated Ca^{++} influx, phosphorylation, desensitization and cellular migration. The results of these experiments should provide insight into the functional roles of various regions of the IL-8 receptors.

I 103 IDENTIFICATION OF INTRACELLULAR DOMAINS IN THE GROWTH HORMONE RECEPTOR INVOLVED IN SIGNAL TRANSDUCTION, Nils Billestrup, Giovanna Allevato and Jens H. Nielsen, Hagedorn Research Laboratory, Gentofte, Denmark

Growth hormone (GH) exerts multiple effects at the cellular level including direct mitogenic actions as well as metabolic and gene regulatory effects. Stimulation of the GH receptor by GH induced dimerization results in all these biological effects; however, the signal transduction mechanism remains largely unknown. Recently it was shown that the tyrosine kinase JAK-2 is likely to be involved in GH receptor signalling and that this kinase can phosphorylate the GH receptor on tyrosine residues. In order to identify functional domains in the GH receptor we have introduced a number of mutations in the intracellular part of the receptor including various C-terminal truncations as well as point mutations. The mutated GH receptors were expressed in various cell lines and the ability of GH to stimulate differentpathways was examined. A GH receptor *lacking* the C-terminal 184 amino acids was deficient in signalling the transcriptional effects of GH on both the insulin and Spi 2.1 genes measured both in stable as well as transient transfection assays; however this mutant was fully active in mediating the effect of GH on MAP kinase activity, JAK-2 kinase activity and GH receptor internalization. Another mutant lacking almost the entire intracellular domain was unable to mediate any effect of GH, but retained high affinity GH binding and cell surface expression. When four proline residues (P 300, 301, 303 and 305) located in a domain of the GH receptor which is highly homologous to other receptors of the GH/erythropoietin/cytokine family (box 1) were mutated to alanine the receptor could no longer mediate the effects of GH on JAK-2 kinase activity, MAP kinase activity and gene transcription but it was still able to internalize normally. When Phenylalanine 346 was mutated to alanine the GH receptor retained all its signalling capabilities, but internalization was greatly reduced. In summary these findings suggest that the GH receptor contains multiple functional domains. The four proline residues present in box 1 define a domain which is crucial for all signalling events studied so far indicating an early activation event. Phenylalanine 346 defines a second domain of importance for high efficiency internalization. The C-terminal 184 amino acids define a third domain which is necessary for transmitting a transcriptional response and we are currently examining the role of tyrosine residues in this domain on the effect of GH receptor mediated gene transcription.

I 104 FUNCTIONAL COUPLING OF SSTR4 TO MULTIPLE SIGNAL TRANSDUCTION PATHWAYS
H. Bito, C. Sakanaka, Z.-i. Honda and T. Shimizu. Dept. of Biochemistry, Faculty of Medicine, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

In order to characterize the molecular mechanism of hippocampal somatostatin receptor (SSTR) signal transduction, we isolated the major hippocampal SSTR cDNA, which was subsequently found to be identical to rSSTR4. Signal transduction pathways coupled to SSTR4 was examined in detail in SSTR4-overexpressing CHO-K1 cells. In these cells, somatostatin-14 not only inhibited forskolin activated-cAMP production, but increased arachidonic acid release, and also activated both p42 and p44 MAP kinases in a dose-dependent and pertussis-sensitive manner. SSTR4, however, was coupled to neither production of IP_3 nor increase in intracellular Ca^{2+}. Further analysis of the signals involved upstream to MAP kinase activation is ongoing. SSTR4-transfected cells will provide a good model for detailed characterization of the heterotrimeric G-protein-dependent pathway of MAP kinase activation.

I 105 SPHINGOLIPIDS MODULATE CALCIUM SIGNALS EVOKED BY EPIDERMAL GROWTH FACTOR (EGF),
Jan Krzysztof Blusztajn*, Peter L. Hudson*†, Ward A. Pedersen*, Mordechai Liscovitch‡, David T. MacLaughlin†, and Patricia K. Donahoe†, *Department of Pathology, Boston University School of Medicine, M1009, Boston, MA 02118; †Pediatric Surgical Research Laboratories, Massachusetts General Hospital, Boston, MA 02114; ‡Department of Hormone Research, The Weizmann Institute of Science, Rehovot 76100, Israel

Receptor-activated breakdown of complex sphingolipids has been identified as a mechanism for generating sphingoid-base-containing putative second messenger molecules whose actions may modulate responses to extracellular signals. Previous studies indicate that the tyrosine kinase activity of the EGF receptor may be modulated by sphingolipids *in vitro*; however, little information is available on the interactions of the sphingolipid second messengers with the EGF-initiated signal transduction cascade *in vivo*. In human epidermoid carcinoma A431 cells EGF caused an increase in $[Ca^{2+}]_i$ as measured by the fluorescent Ca^{2+} binding dye indicator, Indo-1. Sphingosine (1-10 μM), within seconds, markedly enhanced the EGF-evoked Ca^{2+} influx but failed to alter Ca^{2+} release from the intracellular stores and had no effect on Ca^{2+} signals evoked by serum. This effect of sphingosine was not associated with altered EGF receptor protein kinase activity. In contrast, a ceramide, N-acetyl-sphingosine (10 μM), sphingosine-1-phosphate (10 μM), and lysosphingomyelin (10 μM), inhibited EGF-evoked elevations in $[Ca^{2+}]_i$. The modulation of growth factor receptor-regulated changes in $[Ca^{2+}]_i$ may constitute a mechanism by which elevations in cellular levels of specific sphingolipids - which occur transiently upon activation of certain receptors, and chronically in sphingolipid storage diseases - exert respectively their physiological and pathophysiological effects.
Supported by grants from the NIMH MH46095, NIH CA17393 and the US-Israel Binational Science Foundation 89-00392.

I 106 TYPE VIII ADENYLYL CYCLASE: A Ca^{2+}/CALMODULIN STIMULATED ENZYME WITH A POTENTIAL ROLE IN LEARNING AND MEMORY,
James J. Cali, John Krupinski, Geisinger Clinic, Danville PA 17822

The importance of adenylyl cyclase and the second messenger cAMP in neuronal function is underscored by the finding that all eight of the known mammalian forms of this enzyme are expressed in brain (Krupinski *et al*, JBC 267,24858-62,1992). A specific role has been assigned to the Ca^{2+}/calmodulin stimulated form in the processes of learning and memory (Levin *et al*, Cell 68,479-89,1992). In a previous report *in situ* hybridization analysis of rat brain slices revealed a distinctive pattern of type VIII expression, consistent with a functional role in learning and memory (Matsuoka *et al*, J.Neur. 12,3350-60,1992). In an attempt to gain further insight into its role in the brain, cDNAs encoding a full length type VIII adenylyl cyclase from rat brain were isolated using as probe, a type VIII PCR fragment generated previously from guinea pig brain (Krupinski *et al, ibid.*). The cDNAs predict a protein of 1248 amino acids which conforms well to the structural motif proposed for the membrane bound, hormone responsive adenylyl cyclases. By amino acid homology the predicted type VIII protein is most similar to types V and VI. cAMP accumulation in response to hormonal stimuli was measured in polyclonal populations of HEK 293 cells stably expressing a type VIII cDNA construct. Results indicate that the type VIII cDNA encodes a functional adenylyl cyclase which is stimulated by increases in intracellular Ca^{2+}. Assays of membranes prepared from these cells indicate that the Ca^{2+} response is mediated by calmodulin. Type VIII adenylyl cyclase is therefore most similar in activity to type I.

I 107 AN APOPTOSIS DOMAIN IN THE CYTOPLASMIC TAIL OF NGFR HOMOLOGOUS TO CELL DEATH-INDUCING REGIONS OF FAS AND TNFR-I,
Barbara S. Chapman, Department of Neurology, University of California, San Francisco, CA 94143-0114

Programmed or induced cell suicide is an essential architect of developing mammalian nervous, immune and other systems. Death by apoptosis occurs as part of tissue remodeling; cells not destined to function in the completed structure are removed by this mechanism. Cell death by apoptosis can be induced by extracellular signals, or absence of them, in the form of growth factors and cytokines. Known signaling receptors for apoptosis include several members of the low affinity nerve growth factor receptor (LNGFR)/tumor necrosis factor receptor (TNFR) family. These receptors, which have a single trans-membrane-spanning peptide, are related by homologies in their ligand-binding domains. The human Fas antigen (Apo-1) and TNFR-I (Mr 55 K) receptors induce a cell death program in response to ligand binding. The B-cell antigen CD40 and the neurotrophin receptor NGFR (Mr 75 K), can cause cells to depend on ligand for survival, probably by initiating apoptosis when ligand is unavailable. Recently, a cytoplasmic domain was shown to be required for induction of apoptosis by the human Fas antigen, and a homologous region has been reported in the cytoplasmic domain of TNFR-I. Using a matrix homology procedure, I have located this apoptosis motif in residues 350-380 of NGFR, corresponding to Fas antigen residues 246-275 (24% identity) and TNFR-I residues 368-398 (32% matching). Eleven residues at the C-terminus of this motif include the previously described homology to mastoparan, a G-protein activating peptide. Each apoptosis domain appears to contain an amphiphilic alpha helix, suggesting a mechanism for transducing a signal through a G protein-regulated effector. Control of cell survival by NGFR provides a system in which to analyze G-protein coupling to single transmembrane receptors. In addition, understanding this second messenger pathway may help to resolve the complex signals sent by cytokines and growth factors known to elicit various cellular responses, including survival, proliferation and differentiation. For example, although CD40 and NGFR act by promoting cell death in the absence of ligand, their function appears to be modulated by tyrosine-kinase receptors (the antigen receptor complex and trk-family proteins, respectively). Survival and differentiation of neurons may be directed by the interplay of two pathways, a tyrosine kinase cascade initiated by trk receptors, and G-protein coupled second messenger events stimulated by NGFR.

I 108 Direction of coincident cAMP signals can be regulated by the types of adenylyl cyclases present in a cell. *Jiangiang Chen and Ravi Iyengar* Department of Pharmacology, Mount Sinai School of Medicine, CUNY, NY, NY10029

Regulation of cloned type 2(Ac2) and type 6(AC6) adenylyl cyclase by G_i was studied by cotransfection into Cos 7 cells. When Ac2 or Ac6 was cotransfected with $Q205LG_{i2}$-α and LH receptor, hCG stimulated cAMP acumulation was inhibited for both types, although inhibition of Ac6 appeared total while that of Ac2 was partial. Phorbol ester treatment of Cos 7 cells cotransfected with Ac2 and $Q205LG_{i2}$-α and LH receptor resulted in increase hCG stimulated cAMP accumulation and loss of inhibition of Ac2 by $Q205LG_{i2}$-α. Phorbol esters didnot affect stimulation or inhibition of Ac6. The G_i coupled D_2 dopamine receptor elevates cAMP levels in Cos-7 cells. When Cos 7 cells were cotransfected with LH and D_2 dopamine receptors and Ac2, D2 agonists appeared to be more potent in stimulating cAMP production as compared to cells where no foreign adenylyl cyclases were expressed. In contrast, D2 agonists inhibited hCG stimulated cAMP accumulation in Cos 7 cells cotransfected with Ac6 and the D2 and LH receptors. These studies indicate that coincident signals from the G_s and G_i pathways can be integrated at the level of adenylyl cyclase and the direction of the cAMP response is dependent on the types of adenylyl cyclases present.

I 109 IDENTIFICATION AND CHARACTERIZATION OF THE HUMAN CASEIN KINASE I PROTEIN KINASE SUBFAMILY, Erik Christenson, Michael Cicirelli, Anthony J. DeMaggio, Kevin Egan, James B. Hicks, Niki Hoagland, Byron Sebastian, and Merl F. Hoekstra, ICOS Corporation, 22021-20th Ave. S.E., Bothell, WA 98021

Molecular and biochemical analysis of the baker's yeast *HRR25* gene and fission yeast *hhp1*+ and *hhp2*+ genes has revealed that they encode isoforms of casein kinase I. These three forms of CKI play an important regulatory role in DNA metabolism and DNA repair. In order to gain an understanding of how CKI acts in higher organisms, we have isolated and characterized human isoforms of casein kinase I. In this report we will describe our analysis of three CKI isoforms, CKIα, CKIγ, and CKIδ. Molecular cloning and Northern analysis has revealed a complex pattern of expression for the human CKI isoforms. There exists multiple splice variants and all three forms are expressed to varying degrees in all tissues examined.

Unlike the classical literature on CKI, we find that two of the isoforms show a larger molecular mass than typically reported for the enzyme. Indeed, of the known sequence for more than fifteen isoforms of CKI found in yeasts, rodents, and man, only CKIα is close to the typically described mass of 30 - 35 kDa whereas CKIγ and CKIδ have extended carboxy termini. Because of the high degree of amino acid identity within this subfamily of protein kinases, we have examined whether human isoforms are functional counterparts of yeast genes. Complementation of *hrr25* mutants has allowed us to establish that human CKI genes are the functionally equivalent forms of yeast genes. Finally, the expression of recombinant CKIs has greatly facilitated their purification and use for enzymatic characterization. In particular we have examined differences between CKI isoforms in their ability to recognize different substrates and in their susceptibility to different CKI inhibitors. This work reveals that various isoforms show differential substrate recognition and phosphorylation. Finally, isoform-specific antisera have been developed with the goal of determining the subcellular distribution and the regulation of these enzymes. Preliminary experiments reveal that CKIα is localized with cytoskeletal components and that the enzyme distribution reorganizes during cell cycle progression.

I 110 **DISRUPTION OF PDGF RECEPTOR INTRACELLULAR TRAFFICKING BY MUTATION OF ITS PI-3 KINASE BINDING SITES.** Silvia Corvera", Fred Fay"", Andrius Kazlauskas^, Marguerite Joly" ^National Jewish Hospital for Immunology and Respiratory Medicine, Denver, Colorado and Program in Molecular Medicine, Departments of Physiology"" and Cell Biology ", University of Massachusetts Medical School, Worcester, Massachusetts 01655.

The internalization of the platelet-derived growth factor receptor, and of mutants of the PDGF receptor which selectively bind the SH2-containing proteins PI-3 kinase, GAP, PLCγ and Syp, has been analyzed by digital imaging microscopy using an antibody against the exofacial domain of the human type B receptor (PDGFr). At 0°C, both wild type and mutant receptors were visualized in the cell periphery of human HepG2 cells, in a bright, punctate pattern which appeared to correspond to plasma membrane coated pits. Thus, SH-2 containing proteins mentioned above do not appear to be required for the initial clustering of PDGFr at the plasma membrane. Within 10 min of incubation of cells at 37°C, wild type PDGFr internalized into a juxtanuclear region near the Golgi network. A high concentration of internalized receptor was visualized in vesicular structures clustered around the MTOC, which may correspond to lysosomes. In contrast, a PDGFr mutant (F5) which lacks high affinity binding sites for PI-3 kinase, GAP, PLCγ and Syp, remained at the cell periphery. Restoration of the PI-3 kinase binding sites on F5 completely restored the ability of the receptor to concentrate in juxtanuclear vesicles. Like F5, A PDGFr mutant lacking only PI-3 kinase binding sites failed to concentrate in the intracellular juxtanuclear region after 10 min of incubation at 37oC. Thus, PI-3' kinase binding sites appear both necessary and sufficient for the normal intracellular trafficking of the PDGF receptor. These results suggest a role for PI-3' kinase in both signal transduction and receptor sorting in mammalian cells.

I 111 **SIGNAL TRANSDUCTION AND GROWTH REGULATION VIA THE HEPATOCYTE ALPHA$_1$ ADRENERGIC RECEPTOR,** **Jennifer L. Cruise, Tinna Laughlin, Dan Fluegel, and Maureen McDonnell, Department of Biology, University of St. Thomas, St. Paul, MN 55105** The hepatic alpha$_1$adrenergic receptor has been demonstrated to mediate enhancement of DNA synthesis *in vitro* and *in vivo*. It remains unclear whether phosphatidylinositol lipid breakdown is involved in the change in growth response observed with adrenergic stimulation. We investigated the coupling status of alpha$_1$adrenergic receptors in a primary rat hepatocyte culture model in which norepinephrine (NE) significantly enhances DNA synthesis.

We found that receptor coupling, measured as the stimulated production of inositol phosphates (IP) in cells pre-labeled with [^{3}H]-myo-inositol, was lost during the attachment of hepatocytes to collagen-coated plates in the presence of serum. While coupling to lipid breakdown was subsequently restored over time in serum-free media, its reappearance was not correlated with the ability of the receptor to mediate growth enhancement. Other data also failed to support a significant role for IP production: 1) Epidermal growth factor and insulin, at concentrations that stimulated DNA synthesis, stimulated no inositol phosphate production, and their presence reduced IP production via alpha$_1$adrenergic receptors; 2) Vasopressin stimulated more IP production than did alpha$_1$ receptor agonist, but did not enhance hepatocyte DNA synthesis; 3) Neomycin, introduced into primary cultures by transient permeabilization, reduced IP production, but was without effect on the growth response. These data are consistent with a model in which phosphatidylinositol lipid breakdown is not the primary pathway by which the alpha$_1$adrenergic receptor stimulates enhanced DNA synthesis.

Preliminary evidence suggests that while G-proteins may mediate some of the growth-related effects of the alpha$_1$adrenergic receptor stimulus, at least a portion of the response appears to be G-protein independent.

I 112 STRESS-INUCED OVEREXPRESSION OF COUPLED 5HT1A RECEPTORS IN NEURAL CELL LINES, Glyn Dawson, Brett Chromy, Michael Boyers and Probal Banerjee, Depts. Pediatrics and Biochemistry, University of Chicago, Chicago, IL 60637

In order to study the regulation of heptahelical receptor expression under various conditions of environmental stress we used a Rous Sarcoma Virus (RSV) promoter-driven construct, pBCI-G-21, to stably express the serotonin 5-HT_{1A} receptor (5-HT_{1A}-R), coupled to adenylate cyclase inhibition, in three murine neuroblastoma cell lines and also non-neural CHO cells. A strong passage dependent increase of 5-HT_{1A}-R expression was observed only in the HN2 (hippocampal) and NCB-20 (CNS) cells and following continuous culture without feeding, a 3-5 fold increase in agonist binding (5-HT_{1A} sites) was observed during day 10 and 11 in the neural cells (HN2, NCB-20 and F-11) but not in CHO. An accompanying increase in G-21 message level was observed in all cases, whereas actin message declined and G-3-PDH message remained stable. Other stress factors such as hyperammonemia were able to enhance [3H]DPAT binding in both an *in vivo* rat model and HN2 cells. HN2, F-11 and CHO cells were also transfected with a construct, pCMV6-G-21, driven by a Cytomegalovirus (CMV) promoter. Essentially, the same stress induction of 5-HT_{1A}-R expression at day 10 was observed again in both neuroblastoma, HN2 and F-11, transfectants, but not in the non-neural CHO clones, thus confirming that the stress regulation was not due to cis-acting elements present in the RSV promoter used for the first stable expression. Actinomycin D (5 μM) treatment of transfected HN2 cells at day 9 blocked the increase in 5-HT_{1A} sites observed at day 10 without affecting the basal 5-HT_{1A}-R expression, confirming that increased RNA synthesis at day 9 was the reason for this induction. Following Actinomycin D (5 μM) treatment of transfected CHO cells, both the number of 5-HT_{1A} sites and G-21 message decreased by 50% in 6 h whereas no such decrease was observed in the transfected HN2 cells, confirming that the G-21 message was stabilized in the neural cells but not in the CHOs. The 170 base pair G-21 sequence adjacent to the 5'-end of the 5-HT_{1A}-R coding region contains several sites for interaction of stress-activated transcription factors. Therefore, we propose that while stress induction of G-21 transcription could occur in both neural as well as non-neural CHO cells, only the neuroblastoma cell lines contain specific proteins which stabilize the increased G-21 message.

I 113 INTERMODULATION OF NATRIURETIC PEPTIDE RECEPTORS ANF-R_1 AND ANF-R_2 IN ARTERIAL SMOOTH MUSCLE CELLS: ROLE OF PROTEIN KINASE C AND CA^{++}/CALMODULIN-DEPENDENT PROTEIN KINASE, A. De Léan, N. Bouchard, N. McNicoll and L. Larose, Department of Pharmacology, Université de Montréal, Montreal, Quebec, Canada

Vascular smooth muscle cells express multiple natriuretic receptor types including ANR-R_{1A} (ANF-selective), ANF-R_{1C} (CNP-selective) receptors which are guanylate cyclase-coupled and ANF-R_2 receptors (non selective) which appear to be G-protein-coupled. Primary culture of arterial smooth muscle cells in the presence of serum leads to a dramatic shift from a predominantly ANF-R_{1A} phenotype to a predominantly ANF-R_2 phenotype together with significant amounts of ANF-R_{1C}. The simultaneous presence of these receptor types and the balance shift induced by cell proliferation suggests that this balance might be associated with varying pharmacological profiles of natriuretic peptides acting as vascular smooth muscle relaxing and anti-proliferative agents. We assessed the intermodulatory role of ANF-R_2 receptors on ANF-R_{1A} and ANF-R_{1C} in rat aortic smooth muscle cells. The ANF-R_2 specific analog C-ANF induces 75% and 63% desensitization of ANF-R_1-associated cyclic GMP production stimulated by ANF and CNP, respectively. Blockade of protein kinase C by calphostin C completely prevents this desensitization, as observed when desensitization is induced with angiotensin and phobol ester PMA. Prior treatment of smooth muscle cells with pertussis toxin often does not block C-ANF induced desensitization and variably modulates stimulation of cGMP production by CNP. In contrast blocking Ca^{++}/calmodulin-dependent protein kinase with KN-62 inhibits ANF and CNP-stimulated cGMP production by 78% and 80%, respectively. These results indicate that ANF-R_1 receptor-effector coupling is negatively regulated by ANF-R_2 receptor through activation of protein kinase C and ANF-R_1 receptor phosphorylation. The identity of the G-protein involved in mediating ANF-R_2 response is undetermined. In addition, a distinct receptor phosphorylation mechanism involving Ca^{++}/calmodulin-dependent protein kinase is required for positive regulation of guanylate cyclase activation by natriuretic peptides.

I 114 THE AMINO-TERMINUS OF THE HUMAN C5A RECEPTOR IS REQUIRED FOR HIGH AFFINITY C5A BINDING BUT NOT FOR SIGNAL TRANSDUCTION. Julie A. DeMartino, Gail Van Riper, Salvatore J. Siciliano, Zenon D. Konteatis, Hugh Rosen, and Martin S. Springer, Departments of Biochemical and Molecular Pathology, Immunology Research, and Analytical Biochemistry, Merck Research Laboratories, P. O. Box 2000, Rahway, N. J. 07065

The binding domain of the human C5a receptor consists of two distinct and physically separable subsites. One of these sites binds the C-terminal 8 amino acids of C5a and is as yet undefined, while the second site lies in the N-terminus of the receptor and interacts with the core of C5a. Two deletion mutants were prepared to probe the importance of this second site. Removal of residues 2-22 decreased the binding affinity for C5a by 600-fold, while extending the deletion through residue 30 caused a further 75-fold decrease. Thus the N-terminus is responsible for at least 45% of the total energy for the binding of C5a. The five aspartic acids present in the deleted segments appear to be critical residues, as their conversion to alanines accounts for most of the affinity lost in the two truncations. Despite its importance for binding, the N-terminus is not necessary for activation, as both C5a, and C5a C-terminal peptide were able to generate Ca^{+2} fluxes through a receptor lacking residues 2-22.

I 115 EXPRESSION OF Q209L-Gαq POTENTIATES $PGF_{2\alpha}$-STIMULATED GROWTH SIGNALING IN NIH-3T3 CELLS

Michael De Vivo and Ravi Iyengar, Mount Sinai School of Medicine of the City University of New York, Department of Pharmacology, New York, NY 10029.

Clonal NIH-3T3 cell lines expressing a mutant, active form of the G protein Gαq-subunit (Q209L-Gαq) under the control of the MMTV promoter have been isolated. Expression of Q209L-Gαq is induced by dexamethasone. Expression of Q209L-Gαq results in a 5-fold increase in the G protein-coupled receptor agonist $PGF_{2\alpha}$ stimulated inositol phosphate (IP) production. A smaller increase (two-fold) was observed in receptor tyrosine kinase PDGF receptor-stimulated cells. Concomitant with the increase in IP production, $PGF_{2\alpha}$ stimulated DNA synthesis ten-fold greater and PDGF 4 to 5-fold greater in Q209L-Gαq expressing cells. No change in EC_{50} values for either PDGF or $PGF_{2\alpha}$ was detected in Q209L-Gαq expressing cells for either IP production or DNA synthesis. Expression of Q209L-Gαq also doubled $PGF_{2\alpha}$ -stimulated MAP-kinase activity. These results indicate that extensive stimulation of MAP-kinase through the Gαq pathway constitutes a mitogenic signal in NIH-3T3 cells.

I 116 INCREASED PALMITOYLATION OF THE G PROTEIN α_S SUBUNIT AFTER β-ADRENERGIC RECEPTOR ACTIVATION

Michael Y.Degtyarev, Allen M.Spiegel and TeresaL.Z.Jones Molecular Pathophysiology Branch, NIDDK, NIH, Bethesda, MD 20892

The heterotrimeric G_S protein α subunit which couples the β-adrenergic receptor to adenylyl cyclase undergoes palmitoylation. We examined the dynamics of this modification of α_S by metabolic labeling COS and S49 lymphoma cells. The endogenous α_S proteins were immunoprecipitated with a peptide-specific antibody, separated by SDS-PAGE and analyzed by fluorography and densitometry. A pulse-chase study of COS cells incubated with [^{3}H]palmitate or [^{35}S]methionine showed that the turnover of the palmitate moiety ($t_{1/2}$ of approx. 60 min) was significantly faster than the protein degradation. Treatment with 10 uM isoproterenol, a β-adrenergic receptor agonist, in the presence of [^{3}H]palmitate showed a rapid 4-10 fold increase in the palmitoylation of α_S compared to the basal incorporation as early as 2 min. The increase in palmitoylation showed a concentration dependence with a half-maximal labeling at 0.9 uM isoproterenol and could be blocked by the antagonist propranolol. The mutant α_S proteins in the *unc* and H21a S49 cell lines did not show an increase over basal [^{3}H]palmitate incorporation after isoproterenol treatment. The α_S subunits from COS cells pretreated with cholera toxin also showed an increase in [^{3}H]palmitate incorporation. This data indicate that palmitoylation of the α_S subunit is dynamic and regulated by conformational changes produced by activation and suggest a functional role for the modification.

I 117 IDENTIFICATION OF Hrr25p PROTEIN KINASE INTERACTING PROTEINS, Anthony J. DeMaggio and Merl F. Hoekstra, ICOS Corporation, 22021-20th Ave S.E., Bothell, WA 98021

The *S. cerevisiae* gene product Hrr25p, a member of the casein kinase I protein kinase subfamily, has been shown to be involved in DNA repair. *HRR25* was identified through its mutant phenotypes which resulted in *S. cerevisiae* strains that were unable to tolerate DNA double-stranded breaks. Not much is known about the *in vivo* substrates for casein kinase I in yeast or other organisms. We have used the two-hybrid system to identify potential substrates and other proteins that interact with *HRR25*. The genes encoding potential interacting proteins from this screen were designated *HIT1 - HIT5* (*HRR25* Interacting Targets). These hits show specificity for *HRR25* and its *S. pombe* homologue *hhp1*$^+$ while not interacting with other related protein kinases.

A partial *HIT1* fusion gene was used to screen a yeast genomic library to clone the full length *HIT1* gene. DNA sequencing revealed no substantial structural similarity between *HIT1* and previously identified proteins. To determine if *HIT1* was important for any *in vivo* functions, we constructed a *hit1* deletion mutant. Like *hrr25* mutants, *hit1* mutants have aberrant microscopic morphology and impared growth rate. Further more, *hrr25*Δ *hit1*Δ show synthetic lethal inviability. These results suggest that *HRR25* and *HIT1* are important for mediating similar aspects of cell growth and metabolism.

Like *HIT1*, *HIT2* has been cloned and sequenced and shows no homology to other protein in the sequence database. *HIT3* has been evaluated for specificty in interaction and this analysis reveals that it interacts strongly with the *S. pombe* homologue of *HRR25*. Partial sequence of *HIT3* reveals that it is a novel protein.

HIT4 shows weak interaction with full length Hrr25p and sequence analysis reveals that this protein is the COOH-terminal portion of the kinesin-like protein Kip2p. Kip2 has a highly conserved region which contains a kinesin-like microtubule-based motor domain. Because *hrr25* mutants show chromosome segregation defects and because kinesin are important for segregation function. We examined the interaction of several yeast kinesins (*KAR3*, *KIP1*, *KIP2*, *CIN8*) with Hrr25p. These experiments reveal that Hrr25p shows specificity in its interaction with kinesin-like proteins and that kinesins are potential substrates for CKI protein kinases.

I 118 A COMBINATION OF SIGNALS INTERACT TO CONTROL THE ALTERNATIVE SUBCELLULAR LOCALZATION OF FGF3, Clive Dickson, Paul Kiefer, Piers Acland and Gordon Peters, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, UK.

Fgf-3 (formally known as *int-2*) was discovered as a proto-oncogene transcriptionally activated by the action of mouse mammary tumour virus in murine breast tumors. In normal uninfected adult mice, *Fgf-3* expression is restricted to brain and testis, but it shows a complex spatio-temporal pattern in the mouse embryo, suggesting a normal role in mammalian development. The *Fgf-3* gene encodes products with different subcellular localizations which is governed by the selection of alternative initiation codons. Protein initiated at AUG is directed into the secretory pathway by virtue of the N-terminal signal peptide, while FGF3 which initiates at an upstream CUG localizes predominantly to the cell nucleus and nucleolus. An extensive analysis of various mutations has identified a number of interacting motifs that work to effect the subcellular distribution of the FGF3 protein. The amino terminus of the larger, CUG initiated protein, plays a crucial role in directing the protein to the cell nucleus, although it does not contain a classical nuclear localization signal (NLS). A bipartite NLS has been identified within the amino-terminal domain of the protein, downstream of the signal peptide. The amino-terminal region together with the NLS are sufficient to direct a heterolgous protein to the nucleus. In the carboxy-terminal region of the protein are additional signals which are necessary for nucleolar localization. The interplay of at least three protein domains are necessary for the routing of FGF3 to different subcellular locations, providing the potential for alternative signalling pathways.

I 119 Gαq MEDIATED COUPLING OF THE NK2 RECEPTOR TO PHOSPHOLIPASE IS IMPAIRED WHEN Gαq RESIDUES C9 AND C10 ARE MUTATED TO ALANINE, Michael D. Edgerton and Andre Chollet, Department of Biological Chemistry, Glaxo Institute for Molecular Biology, Geneva, Switzerland.

The alpha subunits of several heterotrimeric G proteins, including Gαq, have recently been demonstrated to be modified by the addition of palmitic acid to a cysteine residue located near their amino-terminus. This post-translational modification is found only on G alpha subunits which partition to particulate cellular fractions. To investigate the potential role of palmitoylation in G protein function, we have mutated residues of Gαq likely to be modified with palmitic acid to alanine. Here we show that transient transfection of COS7 cells with plasmids expressing the NK2 receptor leads to an agonist dependant accumulation of diacylglycerol. Agonist stimulation of cells cotransfected with plasmids expressing both the NK2 receptor and Gαq leads to the accumulation of larger amounts of diacylglycerol then in cells expressing the receptor alone, demonstrating an interaction between the NK2 receptor and Gαq. A Gαq protein containing the double mutation C9A,C10A, which removes putative site(s) for palmitoylation, is much less effective at enhancing agonist mediated diacylglycerol accumulation in this assay. As is the case for wild-type Gαq, this mutant Gαq is largely associated with particulate cellular fractions.

I 120 THE USE OF THAPSIGARGIN TO ELUCIDATE THE ROLE OF CALCIUM IN SIGNAL TRANSDUCTION. Mandy Fenton and John E. Kay. Biochemistry Laboratory, School of Biological Sciences, University of Sussex, Falmer, Brighton, BN1 9QG. U. K.

Thapsigargin (Tg) is an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase and at nanomolar levels can be used to manipulate cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). Here Tg is shown to elevate $[Ca^{2+}]_i$ in porcine PBMC with similar kinetics to Con A. It is found to activate PBMC alone and in combination with optimum concentrations of the phorbol ester PMA, but is not always as effective as Concanavalin A (Con A) or PMA/ionomycin. Tg, and Tg/PMA are mitogenic at lower $[Ca^{2+}]_i$ than that produced by mitogenic concentrations of ionomycin. The immunosuppressant FK506, inhibits Ca^{2+} dependent T cell activation via the inhibition of calcineurin phosphatase. Unexpectedly, in contrast to the inhibition of Con A or TPA/ionomycin activation, FK506 enhances activation by Tg/TPA. Also, a 2 hour preincubation with FK506 to eliminate the calcineurin pathway does not inhibit Tg/TPA activation. The effects of Tg on protein kinase activation have also been studied with an increase in protein kinase C and S6 kinase activation apparent within 5-10 minutes of addition to permeabilised PBMC. It is believed that some of these effects are independent of Ca^{2+} and Tg should therefore be used with caution when studying the role of Ca^{2+} in signal transduction.

I 121 TRANSCRIPTION FACTOR p91 MEDIATES DIRECT SIGNALING FROM THE EGF RECEPTOR TO THE c-fos GENE PROMOTER. Xin-Yuan Fu and Jiao-Jiao Zhang, Department of Biochemistry, Mount Sinai School of Medicine, New York, NY 10029

Transcription factor p91 was previously characterized in interferon induced signal transduction and gene expression. p91 contains a SH2 domain and is activated by tyrosine phosphorylation.

Here we demonstrate that epidermal growth factor (EGF) induces rapid tyrosine phosphorylation and nuclear translocation of p91. Through its SH2 domain, p91 directly interacts with EGF receptor in a ligand-dependent manner. p91 is a necessary component of an EGF-induced DNA binding factor that recognizes a previously identified regulatory element, SIE (c-sis-inducible element), in the c-fos gene promoter. Activated p91 stimulates SIE-dependent transcription *in vitro*. Co-transfection of a SIE-containing reporter with a p91 expression vector shows that p91 is a positive transcription regulator of the c-fos gene promoter.

These studies suggest that EGF uses a direct signaling pathway to control nuclear transcriptional events.

I 122 DOMAINS GOVERNING SUBUNIT SPECIFICITY IN $\beta\gamma$ INTERACTION Anja Garritsen and William F. Simonds*
Department of Neuropharmacology, Organon Int., P.O. Box 20, 5340 BH Oss, The Netherlands and * Molecular Pathophysiology Branch, NIDDK, Bethesda MD 20892

The β and γ subunits of heterotrimeric G proteins form a tightly bound complex. Considering the recent progress made in understanding the functional role of the $\beta\gamma$ complex, we set out to elucidate the structural determinants of $\beta\gamma$ interaction. We proposed that one mechanism underlying the assembly of this complex is the formation of a coiled coil like structure between the N terminus of the β subunit (residues 1-30) and the N terminal half of the γ subunit (Proc. Natl. Acad. Sci 90, 7706-7710, *1993*). Not all subtypes of these proteins, however, can combine to form a functional complex. Thus, it was shown by multiple laboratories that in contrast to β_1, β_2 cannot interact with γ_1 although both subtypes interact with γ_2. Since the residues involved in the putative coiled coil-like structure are very well conserved between β_1 and β_2, it seemed likely that other domains might be responsible for selectivity in the assembly of the complex. We have constructed a number of β_1/β_2 chimeras in an attempt to define which of the 33 divergent residues are important for the interaction of β_1 with γ_1. The chimeras were transfected into COS-7 cells in the presence of either γ_1C71S or γ_2C68S. These nonprenylated γ subunits direct the entire $\beta\gamma$ complex to the cytosol. Thus, the appearance of β-immunoreactivity in the soluble protein fraction implies complex formation. All chimeras interacted with γ_2C68S, as expected. Several, however, failed to interact with γ_1C71S , a pattern seen with β_2 as well, or they did so to a much lesser extent than β_1. A complex pattern was found in which several domains seem to play a role in distinguishing the γ subunits. These domains together may form a binding site for γ for which the three-dimensional structural framework could be provided by the WD40-repeats.

I 123 PROBING THE BINDING SITE FOR THE C5a ANAPHYLATOXIN RECEPTOR, Craig Gerard, Bao Lu, and Norma P. Gerard, Department of Medicine, Harvard Medical School, Boston, MA 02115

The C5a anaphylatoxin ligand-receptor interaction on polymorphonuclear neutrophils stimulates chemotaxis, degranulation and the oxidative burst. The receptor is a member of the heptahelical G-protein coupled receptor family. The ligand is a basically charged 72 amino acid peptide derived from the C5 component of complement and has been shown to have structural requirements for association with the receptor which suggest interactions at more than one site. An antibody recognizing the receptor sequence, D_{15}DKDTLD_{21}, can block ligand binding suggesting this region may be a point of association. Mutation of D_{15} and D_{21} to A did not alter binding affinity, however, indicating the charge characteristics are not responsible. In order to definitively determine the role of his region in ligand binding, we replaced the sequence T_{29}SNT_{32} with IEGR, providing a proteolytic site for factor Xa. Exchange of these sequences does not perturb the ligand interaction, and cleavage with factor Xa will remove the entire N-terminal extracellular sequence to allow evaluation of its role in binding ligand. Additional structural requirements have been determined in the third extracellular loop sequence. Mutation of L_{277} to P results in expression of a non-functional receptor; mutation of P_{270} to L behaves like the normal receptor. The effect of introducing a P at this site likely reflects perturbation of the relative orientation of TM6 and TM7, impairing the Gα–receptor interaction, and not a ligand binding point, since addition of P in the analogous position of other receptors whose ligands are structurally different had a similar effect.

I 124 EXPRESSION CLONING OF A cDNA ENCODING A NOVEL MURINE INTERLEUKIN 1 RECEPTOR ACCESSORY PROTEIN, Scott Greenfeder, Richard Chizzonite and Grace Ju, Department of Inflammation and Autoimmune Diseases, Roche Research Center, Hoffmann-La Roche, Nutley, N.J. 07110

A monoclonal antibody was isolated that blocked the binding and bioactivity of both human and mouse IL-1ß on murine IL-1 receptor containing cells. This mAb recognized a protein that was distinct from the Type I and Type II IL-1 receptors, suggesting that a novel protein exists that is involved in IL-1 biological responses. By expression cloning in COS-7 cells, we have isolated a cDNA from mouse 3T3-L1 cells encoding this putative auxiliary molecule, which we term the IL-1 Receptor Accessory Protein (IL-1RAP). Sequence analysis of the cDNA ORF predicts a 570 amino acid protein with a molecular weight of ~66kd. The protein sequence predicts a 20 amino acid signal peptide and a 29 amino acid transmembrane domain that divides the protein into a 340 amino acid extracellular and 181 amino acid cytoplasmic domain. The location of the signal peptide cleavage has been confirmed by N-terminal sequence analysis of natural IL-1RAP purified from murine EL-4 cell membranes. IL-1RAP has limited homology to both the Type I and Type II IL-1 receptors of mouse, human, chicken and rat. The homology is uniformly distributed throughout the protein; however, the cysteines responsible for formation of three IgG-like domains in the IL-1 receptors are perfectly conserved in IL-1RAP. No significant homology to other proteins in either Genbank or the Swiss-Protein database was found. Northern analysis reveals a homologous 5.3kb mRNA in brain, lung, spleen and thymus that appears to be increased by IL-1 treatment. A 1.8kb mRNA is found in liver that appears to be decreased by IL-1 treatment. No mRNA is found in kidney. Functional studies of IL-1RAP are in progress.

I 125 IDENTIFYING THE SITE OF PHOSPHORYLATION ON Gα2 OF *DICTYOSTELIUM*. Robert E. Gundersen, Mei-Yu Chen*, and Peter N. Devreotes*, Department of Biochemistry, Microbiology, and Molecular Biology, University of Maine, Orono, ME 04469, *Department of Biological Chemistry, The Johns Hopkins University Medical School, Baltimore, MD 21205.

In eukaryotic cells G proteins couple cell surface receptors to response-producing effectors inside the cell. Their signal transducing function is essential in a wide variety of eukaryotic cell processes, including chemotaxis and differentiation in *Dictyostelium discoideum*. Regulation of G proteins appears to reside solely in their rate of GTP hydrolysis. However G protein α-subunits have been reported to be phosphorylated *in vivo* in hepatocytes, platelets, and *Dictyostelium*. In *Dictyostelium* phosphorylation occurs on the α-subunit, Gα2; is dependent on cAMP binding to surface cAMP receptors; and occurs on serine(s). The function of α-subunit phosphorylation remains obscure. In order to gain insight into the role of α-subunit phosphorylation we identified the site of Gα2 phosphorylation as serine-113, by using 2D (IEF/SDS) gel electrophoresis, peptide mapping, construction of α-subunit chimeras, and site-directed mutagenesis. Two dimensional gel electrophoresis reveals an isoelectric point for Gα2 of approximately 7.5-7.7 in 9.5 M urea, while the phosphorylated Gα2 exists as a single spot shifted approximately 2 charge units to the acidic end. This is consistent with the addition of a single phosphate. Gα2/Gα1 and Gα1/Gα2 chimeras were used to isolate the Gα2 sequence required for phosphorylation. Results from the Gα2/Gα1 chimeras suggest a site between amino acids 101 and 215, while the Gα1/Gα2 chimeras limit the site to between amino acids 33 and 154. Peptide mapping demonstrated that phosphorylation occurred within the first 119 amino acids, thus limiting the site to amino acids 101 to 119. This sequence contains two serine residues Ser-109 and Ser-113. Site-directed mutagenesis was used to individually change these two serines to alanine. Ser-113 fails to be phosphorylated upon cAMP stimulation yet can undergo aggregation and differentiation. Confirmation of Ser-113 as the site of Gα2 phosphorylation is being pursued. Identification is being followed up by analysis of the phenotype and biochemistry to establish the role of Gα2 phosphorylation.

I 126 **CCK_B-INDUCED ACTIVATION OF AP-1 IN THE EXOCRINE RAT PANCREAS**, Rainer Günther, Iris Bauer, Klaus Domagk, Wolfgang E. Schmidt, I. Department of Medicine, Laboratory for Molecular Gastroenterology, University of Kiel, 24105 Kiel, Germany.

The exocrine pancreas is the major site of digestive enzyme production which is under both short- and long-term neuronal and hormonal control. The mechanisms responsible for short-term regulation of the pancreas and in particular stimulus-secretion coupling, have been extensively studied. In contrast, the elements involved in the long-term adaptive response have not yet been identified. Recent studies demonstrated the induction of c-fos and c-jun mRNA in the rat pancreas. To evaluate the physiological role of this induction, the regulation of the AP-1 complex was characterized by gel retardation assays. Stimulation of rat pancreatic lobules and the rat pancreatic carcinoma cell line AR42J with CCK_8 induced AP-1 activity. This induction was rapid, transient and concentration-dependent. Detectable stimulation in lobules was seen at 10^{-10}M, in AR42J cells at 10^{-8}M . The CCK_A-receptor antagonist loxiglumide abolished CCK_8 stimulated AP-1 activity. The CCK_B-receptor agonist gastrin induced the AP-1-complex only at high molar concentration (10^{-5}M). Further data will demonstrate some elements involved in the signal-transduction cascade between the CCK_A receptor and AP-1.

Our results suggest that physiological concentrations of CCK_8 stimulate AP-1 binding activity via the CCK_A receptor, whereas the targets of the AP-1 complex in the rat pancreas are still unknown.

I 127 POINT MUTATIONAL STUDIES OF A PUTATIVE DISULFIDE BOND IN THE THYROTROPIN RECEPTOR, Bengt Gustavsson, Nils-Erik Heldin and Bengt Westermark, Department of Pathology, University Hospital, S - 751 85 Uppsala, Sweden.

The TSH receptor is a member of the seven transmembrane spanning (7-TMS) receptor family, and is coupled to the $G_s\alpha$/adenylate cyclase signaling system. A few studies have been made concerning the importance of specific regions as well as single amino acids in the TSH receptor molecule, with regard to ligand binding and adenylate cyclase activation.

In the present study we have focused our interest on two of the thirteen cysteines located in the extracellular part of the receptor, viz. cysteines C494 and C569, located in the first and second extracellular loop, respectively. Suggestions have been made, that in the wild type receptor, there is a disulfide bond between these two amino acids that plays a role in its ability to bind ligand and to stimulate adenylate cyclase activity.

By using the in vitro mutagenesis system Altered Sites™, three different Cys -> Ser mutants of the thyrotropin receptor were created, viz. the two single mutations C494S and C569S, and the double mutation C494S/C569S. The corresponding cDNAs were inserted into the eukaryotic expression vector pSV7d and transiently expressed in transfected COS cells. Seventytwo hours after transfection, the COS cells were assayed with regard to ligand binding and ligand-stimulated cAMP formation. Binding assay with ^{125}I-labeled TSH showed a relatively high binding to the wild type receptor. TSH did not bind to the C569S receptor, and showed only a marginal binding to the C494S receptor. However, a significant binding to the C494S/C569S receptor was obtained, although the binding capacity of the double mutant was reduced, as compared with the wild type receptor. The only receptor variant that was capable of stimulating adenylate cyclase activity in response to TSH, was the wild type receptor. Thus, none of the mutated receptor variants was able to mediate cAMP formation. Ligand binding experiments were also performed with patient thyroid stimulating autoantibodies (TSAb:s), indicating that the major binding site for TSAb:s may be situated in the N-terminal, extracellular part of the receptor: Preincubation with TSAb:s for 1 hour completely abolished TSH binding of the doubly mutated receptor but not the wild type receptor. In conclusion, the present data suggest that the putative disulfide bond between the two cysteines C494 and C569 in the thyrotropin receptor, is required for its signaling function in response to TSH, but not obligatory for TSH binding. Single mutations (C494S and C569S) may allow for illegitimate disulfide bonding, or affect processing and cell surface expression of the receptor.

I 128 **LOCALIZATION OF THE γ_5 SUBUNIT OF SIGNAL TRANSDUCING G PROTEINS TO REGIONS OF FOCAL ADHESION.** Carl A. Hansen, Allen Schroering, David Carey and Janet D. Robishaw. Geisinger Clinic, Weis Center for Research, Danville, PA 17822.

Signal transducing heterotrimeric G proteins are responsible for coupling a large number of cell surface receptors to the appropriate effector(s). Of the 3 subunits, 16 α, 4 β and 5 γ subunits have been characterized. While evidence for additional subunits is clear, the known subunits provide a potential for over 300 unique combinations of heterotrimeric G proteins. To begin deciphering the G protein combinations that couple specific receptors with effectors, we examined the subcellular localization of the γ subunits. Using anti-peptide antibodies specific for each of the known 5 γ subunits, neonatal cardiac fibroblasts were screened by standard immunocytochemistry. The γ_5 specific anti-peptide antibody yielded a highly distinctive pattern of intensely fluorescent regions near the periphery of the cell that tended to protrude into the cell in a fibrous pattern. Dual staining with an anti-vinculin antibody and phalloidin indicated that γ_5 and vinculin co-localized and that the γ_5 immunofluorescence extended a short distance out from the vinculin pattern along the protruding stress fibers. These data indicated that γ_5 was localized to areas of focal adhesion. Dual staining of rat aortic smooth muscle cells and schwann cells also resulted with co-localization of γ_5 and vinculin, suggesting that the association of γ_5 with areas of focal adhesion was widespread. *Supported by NIH grants HL 49278 and GM 39867.*

I 129 VISUALIZATION OF N-FORMYL-PEPTIDE RECEPTOR INDUCED ACTIVATION OF GI PROTEIN IN HL 60 MEMBRANES

Cobi J. Heijnen, Frank Jeurissen, Robert G. Feldman, Annemieke Kavelaars
Dept. Immunology, University Children Hospital, Utrecht, The Netherlands

The association of specific G proteins with particular receptors, and thereby the regulation of specific effectors, is receiving detailed attention at present. Most data are derived from reconstitution experiments, in which recombinant or purified proteins were used. We studied N-formyl-peptide receptor activation of GTP-binding proteins in native HL-60 membranes. In this system we utilized the property of GTP binding proteins to irreversibly bind the non-hydrolysable GTP analog GTPγ^{35}S in the presence of $MgCl_2$. We show that N-formylmethionyl-leucyl-phenylalanine (fMet-Leu-Phe) induces a 2 to 8 fold increase in GTPγ^{35}S incorporation in three major proteins or protein complexes of 50 kD, 115 kD, and 240 kD. The binding of GTPγ^{35}S to these bands could be displaced by unlabelled GTPγS, indicating that the binding was specific. The appearance of the three bands altered with increasing concentrations of GTPγS. Whereas the 115 kD complex was predominant at 10^{-8}M GTPγS, the 50 kD and the 240 kD polypeptides were predominantly labelled at $3x10^{-10}$M GTPγS. Immunoblotting with anti-Gαi1,2 antibody revealed three bands corresponding to 50, 115, and 240 kD. We conclude that the 50 KD is the free Gαi-2 subunit. The identity of the 115 kD and 240 kD polyproteins remains to be determined.

I 130 NUCLEAR CAM KINASE REQUIRES A LOWER STIMULUS THRESHOLD FOR ACTIVATION THAN CYTOPLASMIC CAM KINASE, E. Kevin Heist, Tracey Ollick and Howard Schulman, Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA 94305

Cellular processes such as gene transcription and cell cycle progression are believed to be controlled by calcium/calmodulin-dependent phosphorylation and dephosphorylation reactions occurring in the nucleus. Very little is known, however, about the ability of calcium-mobilizing stimuli to activate calcium/calmodulin-dependent protein kinases and phosphatases in the cell nucleus. Recently, an isoform of the multifunctional calcium/calmodulin-dependent protein kinase (CaM kinase) has been identified and cloned which targets specifically to the nucleus in a number of cell lines tested, in contrast to the cytosolic localization of other isoforms of this kinase.

This report describes experiments comparing activation of nuclear vs. cytoplasmic CaM kinase transfected into PC12 cells in response to several calcium-inducing stimuli. We have found that stimuli which cause a moderate increase in intracellular calcium activate nuclear CaM kinase significantly while causing little or no activation of cytoplasmic CaM kinase. Stimuli which cause greater increases in intracellular calcium activate both forms of the kinase to nearly equal levels, however. Confocal fluorescent microscopy utilizing the intracellular calcium indicator Fluo-3 demonstrates uniform calcium levels prior to stimulation in these cells. After stimulation, however, calcium reaches greater peak levels in the nucleus than in the cytoplasm. These results demonstrate that CaM kinase can be activated in the cell nucleus at a stimulus threshold below that required for activation of cytoplasmic CaM kinase, possibly related to increased levels of calcium in the nucleus vs. the cytoplasm of these cells.

I 131 TSH-INDUCED RECEPTOR INTERNALIZATION IN NON-THYROIDAL CELLS TRANSFECTED WITH A HUMAN TSH RECEPTOR cDNA. Nils-Erik Heldin, Bengt Gustavsson, Annika Hermansson and Bengt Westermark, Dept. of Pathology, University Hospital, S-751 85 Uppsala, Sweden.

Thyroid cells rapidly lose their ability to respond to repeated TSH-stimulations. The mechanism(s) behind this desensitization phenomenon is not clearly understood. In the present investigation desensitization was studied using nonthyroidal cells stably transfected with a human TSH receptor (TSHR) cDNA.

Mouse NIH 3T3 cells and Chinese hamster ovary (CHO) cells were transfected with a human TSHR cDNA expression vector denoted pSV7d-TSHR-WT. Preincubation of the NIH 3T3-TSHR cells resulted in a decreased response to a second TSH-stimulation measured as formation of cyclic AMP (cAMP). Half-maximal inhibition was observed after 4-6 hours with TSH, and the exposure of cells to TSH for 20 hours led to a 80-90 % inhibition of cAMP formation. In order to measure the ligand-induced down-regulation of TSH-receptors, cells were preincubated with TSH and subjected to an acid wash to remove the ligand still bound to receptors present on the cell surface after the preincubation. Half-maximal decrease in the binding of ^{125}I-TSH was detected after 4-6 hours of incubation with TSH. Neither receptor downregulation nor desensitization measured as cAMP formation, could be mimicked by forskolin and therefore not mediated by cAMP.

In contrast to our findings on NIH-3T3-TSHR cells, addition of TSH to CHO-TSHR cells did not lead to a decreased response to a second TSH-stimulation. Moreover, binding of ^{125}I-TSH to the CHO-TSHR cells was not affected to any higher degree by the addition of TSH.

In summary, the homologous desensitization observed in the TSHR-transfected NIH 3T3 cells is probably the result of ligand-induced receptor internalization, since there was a nearly perfect correlation between the binding of ^{125}I-TSH and responsiveness to TSH. In comparison to the NIH-TSHR cells, the CHO-TSHR cells showed no receptor down regulation and desensitization, despite a more efficient endocytosis of receptor-bound ligand. This discrepancy indicates that the TSH receptor is recycled to higher extent in the CHO cells compared to the NIH 3T3 cells, or there is a very rapid synthesis of new receptors replacing the down-regulated receptor.

I 132 GENE TARGETING OF GAP

Mark Henkemeyer, Derrick Rossi, Alan Bernstein, Janet Rossant, and Tony Pawson. Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, 600 University Ave., Toronto, Canada.

Biochemical studies indicate the $p21^{ras}$-GTPase Activating Protein (Ras-GAP) functions to negatively regulate the activity of Ras. GAP is also implicated to function downstream of Ras and thus, may serve as an effector or downstream component of Ras pathways. In addition, the presence of SH2 domains and tyrosine phosphorylation sites in GAP further suggests this protein may receive signals from tyrosine kinases. Very little is known about the cell signaling functions of GAP in normal growth and development, therefore, we have chosen to perform a genetic analysis of its function in mice. The gene encoding GAP (*Gap*) has been cloned and successfully mutated in embryonic stem cells (ES). Chimeric mice for *Gap* heterozygous ES cell lines have been obtained and bred to determine those capable of germline transmission. The initial analysis of *Gap* homozygous offspring and preliminary biochemical studies with *Gap* homozygous ES cell lines will be presented

I 133 MOLECULAR CLONING AND STRUCTURAL ANALYSIS OF THE MURINE CALCITONIN RECEPTOR GENE

Holden, K. S. and Hobart, P. M., Department of Molecular Genetics and Protein Chemistry, Central Research Division of Pfizer, Groton, CT 06340.

The peptide hormone calcitonin elevates serum Ca++ levels by targeting osteoclast dependent bone metabolism via a unique plasma membrane receptor based cell activation. This receptor represents one of the few known genes specifically expressed in the highly differentiated and infrequent mammalian osteoclast. With an interest in developing an osteoclast specific expression system, we have isolated genomic DNA fragments encoding the murine calcitonin receptor (mCTR) using cDNA clones derived from calcitonin receptor mRNA expressed in both brain and kidney tissue. A series of three overlapping 70 kb genomic fragments (cloned in P1 Bacteriophage) have been used to identify this gene, estimated to be over 100 kb in size. Mapping the 5' the transcriptional start site using the RACE technique has revealed two introns in the 5' untranslated (UT) region of the mCTR mRNA. Based on this information, subcloned fragments representing up to 3.6 kb of the presumptive mCTR gene promoter DNA, have been used to direct in vitro cell specific expression of a reporter (Chloramphenicol Acetyl Transferase) gene and thereby indicating their role in regulating the mCTR gene. Using 5' RACE to map the 5' end of the mRNA, we have determined that there are two introns in the gene between the transcriptional and translational start sites. Structural differences in the 5' untranslated sequence of the murine brain and kidney mCTR cDNAs, when compared to cloned genomic DNA, suggests that differential splicing of these two 5' untranslated region introns may be involved in differential expression of this gene in tissues. Comparison of the predicted sequence of the murine mCTR proteins (kidney and brain) with that predicted from human, porcine, and rat cDNA clones indicates the amino terminal domains, including the major extracellular ligand binding domain, are not highly conserved. Indeed, the murine receptor contains an 83 residue extracellular domain segment not found in the human, porcine, or rat cDNAs.

I 134 *Abstract Withdrawn*

I 135 IDENTIFICATION OF STRUCTURAL ELEMENTS IN THE PTH/PTHrP RECEPTOR CRITICAL FOR G-PROTEIN COUPLING.

Zhengmin Huang, Ying Chen, Tom Bambino, Stacy Pratt, Tsui-Hua Chen, Dolores Shoback and Robert A. Nissenson. Endocrine Unit, VA Medical Center, Dept. of Medicine and Physiology, UCSF, San Francisco, CA 94121

High affinity agonist binding, adenylate cyclase (AC) activation and intracellular calcium (Ca^{++}) mobilization by the parathyroid hormone (PTH)/ PTH-related protein (PTHrP) receptor requires coupling to G-proteins. To identify structural determinants in the opossum kidney (OK) PTH/PTHrP receptor critical for signaling, we utilized tandem alanine scanning mutagenesis to replace stretches of four consecutive amino acid residues with alanines. Seven mutant receptors, which scanned the entire third intracellular loop (IC-3), were constructed and transiently expressed in COS-7 cells. To our surprise, most of the mutant receptors retained high affinity bPTH(1-34) binding and AC stimulation indistinguishable from that of the wild type (WT) receptor. However, two mutation stretches in the juxtamembrane regions of IC-3 (one on each end) resulted in PTH receptors with WT PTH binding affinity (IC_{50}= 1nM) but with reduced efficiency of coupling to AC (EC_{50}= 1.3nM vs. WT EC_{50}= 0.1nM). To assess the function of the 127 aa C-terminal tail (IC-4), we constructed a series of mutant OK PTH/PTHrP receptors with progressive C-terminal truncations and expressed them in COS-7 cells. Receptors with a tail as short as 16 residues retained high affinity PTH binding and AC activation similar to WT. However, Receptors with an 8-residue tail displayed decreased PTH binding (20% of WT) and slightly decreased AC (70% of WT). Complete truncation of IC-4 resulted in a loss of both functions. Immunocytochemistry revealed that WT receptors were expressed diffusely in transfected COS cells, whereas mutant receptors lacking IC-4 displayed distinct perinuclear localization. The role of the IC-4 in PTH-induced Ca^{++} mobilization was assessed in Xenopus oocytes injected with PTH receptor cRNAs using a $^{45}Ca^{++}$ efflux assay. PTH stimulated $^{45}Ca^{++}$ efflux in WT and mutant receptors bearing an 8-residue tail, however, no response was seen with mutant receptors lacking the tail. In summary: 1) most side-chains in IC-3 of the PTH/PTHrP receptor are not essential for high affinity PTH binding or AC activation; 2) side-chains from 8-residues in the juxtamembrane regions of IC-3 contribute to activation of AC but may not be required for receptor-G-protein-association *per se*; 3) most of the C-terminal tail is not required for high affinity PTH binding, AC activation, or Ca^{++} mobilization; and 4) a small portion of the tail adjacent to the membrane may contain sequence required for proper expression and function of the PTH/PTHrP receptor.

I 136 THE CHARACTERISATION OF SIGNALLING PATHWAYS INVOLVED IN EPSTEIN-BARR VIRUS (EBV) LATENT MEMBRANE PROTEIN-1 (LMP-1) FUNCTION: IDENTIFICATION OF THE REGIONS REQUIRED FOR NF-κB ACTIVATION

David S. Huen[1], Sheila A. Henderson[1], David Liebowitz, Sally Leevers, Fernando Arenzana-Seisdedos, Elliot Kieff, Chris Marshall, Alan B. Rickinson[1] and Martin Rowe[1].
[1]Institute of Cancer Studies, The Medical School, Birmingham B15 2TJ, U.K.

The gammaherpesvirus Epstein-Barr virus efficiently transforms human peripheral blood lymphocytes *in vitro* to immortalised lymphoblastoid cell lines. Other studies have shown that the virally encoded latent membrane protein-1 is essential for immortalisation activity and also induces morphological transformation of some rodent fibroblast lines in vitro. More recently, LMP-1 has been demonstrated to induce NF-κB activity in a range of cell lines.

LMP-1 consists of a six putative transmembrane helices flanked by a short amino-terminal domain and a large carboxy-terminal domain, both of which are cytoplasmically located. We have studied the structural requirements of NF-κB activation with a panel of LMP-1 mutants and demonstrate that substantial deletions can be made in both cytoplasmic domains without abolition of NF-κB activation whereas deletions within the transmembrane domains ablate NF-κB activation completely.

We shall also present our investigations into the biochemistry of LMP-1 action with particular reference to the NF-κB, AP-1 and MAP kinase pathways.

I 137 D2 DOPAMINE RECEPTORS STIMULATE MITOGEN ACTIVATED PROTEIN KINASES,

Rita M. Huff and Mary E. Lajiness, Cell Biology, Upjohn Laboratories, Kalamazoo, MI 49001

Agonists acting at D2 dopamine receptors, members of the G-protein linked family of receptors, trigger the activation of multiple signalling events. The types of signals which are activated are dependent on the cell type. In CHO cells, D2 receptors inhibit cAMP formation, potentiate ATP-stimulated arachidonic acid release, and stimulate Na^+/H^+ exchange. In addition dopamine, acting through D2 receptors potently stimulates mitogenesis through a pathway which was determined to be independent of the previously mentioned signalling events. Recently we found that D2 receptors trigger a rapid stimulation of phosphorylation on tyrosine residues of a number of proteins including erk1 and erk2. Mitogen activated protein kinase (MAPK) enzyme activity in cell lysates is markedly enhanced by prior treatment of the cells with dopamine. Dopamine-stimulated MAPK activity does not occur in cells which do not express D2 receptors and is blocked by D2 receptor antagonists. The dose response curves for dopamine-stimulated MAPK enzyme activity and dopamine-stimulated mitogenesis are superimposable. In addition, dopamine-mediated stimulation of MAPK is blocked by pertussis toxin pretreatment, indicating that signal transduction occurs through a G protein. PMA treatment of CHO cells also increases MAPK enzyme activity in cell lysates, although to only 75% of the activation by dopamine. Although D2 dopamine receptor-stimulated arachidonic acid release is mediated through a PKC-dependent pathway, downregulation of PKC only partially reduces dopamine stimulated MAPK activity indicating that D2 receptors utilize at least two pathways to activate MAPK.

I 138 CHARACTERIZATION OF Gs COUPLING TO β-ADRENERGIC RECEPTOR AND ADENYLYLCYCLASE IN THE HEARTS OF TRANSGENIC MICE OVEREXPRESSING Gsα,

Yoshihiro Ishikawa, Christophe Gaudin, David C. Wight, Dorothy E. Vatner, Charles J. Homcy, Columbia University, New York, NY, New England Regional Primate Research Center, Southborough, MA, and Medical Research Division of American Cyanamid Company, Pearl River, NY.

Evaluation of the stoichiometry of the signaling components, such as Gsα, has been difficult to assess in the heart since cultured myocytes show resistance to transfection and overexpression of foreign gene products. To overcome this problem, we have developed transgenic mice overexpressing Gsα in the heart using α-MHC promoter, a heart-specific promoter, and examined whether the coupling protein Gsα is a rate limiting component within this signal transduction pathway. Gsα protein levels were increased selectively in the hearts of transgenic mice (T), up to 2.8 times the control levels (C). Gs activity, as assessed by reconstitution in S49 cyc- cell membranes, was also increased (T=4.7±0.4; C=3.2±0.3 pmol cAMP/mg protein 15 minutes; mean±SEM; $p<0.01$). Adenylylcyclase activity in crude sarcolemmal membranes was not altered, either under basal (T=163±74; C=146±56 pmol/mg protein/15 minutes; n=8) or stimulated conditions (GTP + isoproterenol, GTPγS, NaF and forskolin). However, the ratio of high affinity/low affinity agonist binding sites was significantly increased (T=73/27±7.4%; C=55/45±4.2%; $p<0.04$). These results indicate that myocardial Gs levels are already functionally saturating relative to adenylylcyclase but not in their coupling with β-adrenergic receptors to form high affinity agonist binding sites.

I 139 MOLECULAR CLONING OF A NOVEL ALPHA SUBUNIT OF G PROTEIN IN HAMSTERS,

Hui Jin and Harvey Moldofsky, Centre for Sleep and Chronobiology, University of Toronto, Toronto Hospital, Western Division, Toronto, Ontario, Canada M5T 2S8

The identity of individual heterotrimeric G proteins is currently defined by the alpha subunit. More than 18 alpha subunits have been cloned in mammals. We report here the discovery of yet another alpha subunit of G protein in mammals. In the pursuit of mammalian homologue(s) of *Drosophila* rhythm gene (*per*), a pair of degenerate oligos were designed on the basis of exon 5. Hamster genomic DNA was amplified by polymerase chain reaction (PCR) using the degenerate primers, and PCR products were subcloned and sequenced. One of the clones, Clone 77, contains an open reading frame. The nucleotide sequence and deduced amino acid sequence are, 92% and 96% respectively, homologous to the corresponding region of alpha-13 cloned in mouse tissue. Thus, Clone 77 seems to be a new member of G12 subgroup of the family of heterotrimeric G proteins. This novel alpha subunit is very likely a species homologue of mouse alpha-13, and has a similarly ubiquitous tissue distribution. The expression of Clone 77 can be detected in kidney, liver, heart, muscle and brain in hamsters.

I 140 CALCINEURIN PARTICIPATES IN THE NF-*k*B SIGNAL TRANSDUCTION PATHWAYS

Tomohiko Kanno, Guido Franzoso, Keith Brown and Ulrich Siebenlist.
Laboratory of Immunoregulation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda MD 20892

Stimulation of T cells by mitogens results in the rapid activation of transcription factors such as NF-*k*B. Immediately after PHA stimulation, the induced NF-*k*B complex consists of p50/p65 heterodimers, whereas during later stages p50/c-rel appears in a cycloheximide-insensitive manner. Cyclosporin A, which targets calcineurin, blocks rapid induction of p50/p65 by PHA, and partially blocks p50/c-rel induction. These data suggest that calcineurin takes part in the activation of NF-*k*B mediated by PHA. To dissect the NF-*k*B activation pathways further, we have established a Jurkat cell line stably transfected with constitutively active form of calcineurin (CNΔCaM-AI/Jurkat). CNΔCaM-AI/Jurkat showed no constitutive NF-*k*B binding activity without any mitogen treatment. When stimulated with PMA, CNΔCaM-AI/Jurkat induced NF-*k*B at lower concentrations, and NF-*k*B binding persisted for longer periods when compared to a control cell line. Prolonged NF-*k*B binding seen in CNΔCaM-AI/Jurkat following PMA treatment was abolished by cyclosporin A. Taken together, calcineurin appears to play a role in NF-*k*B induction both during early and late stages, but is not sufficient and requires additional components.

I 141 SUBSTANCE P SIGNAL TRANSDUCTION IN HUMAN MONOCYTES: ACTIVATION OF MONOCYTES VIA A NON-NEUROKININ SUBSTANCE P RECEPTOR THAT IS COUPLED TO GI PROTEIN, CALCIUM, PHOSPHOLIPASE D, MAP KINASE AND IL-6 PRODUCTION

Annemieke Kavelaars[#], Frank Jeurissen[#], Richard Franklin[*], Erwin W. Gelfand[*] and Cobi J. Heijnen[#]
[#]Dept. Immunology, University Children Hospital, Utrecht, The Netherlands, [*] Dept. Basic Sciences, National Jewish Center for Immunology, Denver, CO

Substance P (SP) is a tachykinin that is involved in the regulation of inflammatory processes. Tachykinins bind to three subtypes of neurokinin receptors with differences in the affinity for each of the subtypes. We demonstrate that monocytes express a SP binding site, that is not one of the known neurokinin receptors. Triggering of this non-neurokinin SP receptor results in the induction of Interleukin-6 production by monocytes. We also demonstrate that this novel SP binding site is coupled to a Gi protein, by the fact that the amount of GTP-γ-S bound to Gi is increased after activation of monocyte membranes with SP. Triggering of this novel SP binding site leads to transient activation of phospholipase C and a rapid rise in cytosolic calcium. In a *more sustained* way, SP stimulates phospholipase D activity in human monocytes. Further downstream SP gives rise to the activation of MAP-kinase in monocytes. All of the effects of SP can be blocked by pretreatment of the cells with pertussis toxin, which supports our finding that the cascade of second messengers is initiated via coupling of the receptor to Gi.
The order of potency for activation of signal transduction pathways for various ligands is directly related to the Ki for displacement of labeled SP by these ligands. Therefore, our data strongly suggest that all of the effects of SP reported here are mediated via the novel SP receptor.

I 142 Association of the Erythropoietin Receptor with Phosphatidylinositol-3-Kinase and Type II Phosphatidylinositol-4-Kinase

Ursula Klingmüller[1,2], Harvey Lodish[2] and Lewis Cantley[1]
[1]Beth Israel Hospital c/o Harvard Medical School, Division of Signal Transduction, 200 Longwood Avenue, Boston MA 02115; [2]Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge MA 02142-1479

Signalling through the erythropoietin receptor (EPOR) regulates proliferation, differentiation and survival of red blood cells. Being a member of the large family of hematopoetic cytokine receptors, the EPOR lacks intrinsic tyrosine kinase activity in its cytoplasmic tail. The mechanism of signal transduction used by the EPOR is poorly understood.
To address the question, whether phosphatidylinositol (PtdIns) kinases are involved, we conducted coimmunoprecipitation experiments. Analysis of EPOR immunoprecipitats from BaF3 cells expressing wildtype EPOR by Western blotting revealed an association with p85, the regulatory subunit of PtdIns-3-kinase. To test for ezymatic activity the immunocomplexes were assayed for phosphorylation of PtdIns. As expected for PtdIns-3-kinase activity the production of PtdIns-P was reduced 50 to 90% by the addition of detergent. HPLC analysis of the lipids showed that besides a major amount of PtdIns-3-P also PtdIns-4-P was formed, indicating an association of the EPOR not only with PtdIns-3-kinase, but also with an PtdIns-4-kinase. Experiments where GST fusion proteins of the EPOR cytoplasmic tail were incubated with cytosolic extracts revealed only the association with type II PtdIns-4-kinase. These findings could be explained by a direct and constitutive association of the EPOR with PtdIns-4-kinase, while the interaction with PtdIns-3-kinase possibly depends upon ligand induced phosphorylation. To evaluate this hypothesis tyrosine residues in the cytoplasmic domain of the EPOR were mutated. When tested in BaF3 cells, none of the mutated receptors was impaired in mitogenic signalling or association with PtdIns-3-Kinase. This results suggest that the assocation of EPOR with PtdIns-3-kinase either involves the simultanous interaction with several phospotyrosines or with adaptor proteins.

I 143 G-protein–coupled Receptors: Do They Share a Common Ancestor?, Lee F. Kolakowski, Jr. and Kenneth A. Rice, Renal Unit, Massachusetts General Hospital and Harvard Medical School, Charlestown, MA 02129 and Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138.

G-protein–coupled receptors, G-proteins and intracellular effectors are components of a modular system linking extracellular signals to intracellular second messengers. The receptor super-family can be classified into five families, each of which share structural and perhaps physiological characteristics. The classification is derived from analyses of the protein sequences using an accepted mutation parsimony method. The five families are: 1) rhodopsin and β2-adrenergic receptor-like sequences; 2) PTH/calcitonin/secretin receptor-like sequences; 3) metabotropic glutamate receptor-like sequences; 4) fungal pheromone receptor-like sequences; and 5) *D.discoidium* cAMP receptor-like sequences. Evidence from cloning work in *C. elegans* and Jellyfish show that both the family#1 and family#2 above existed simultaneously through most of metazoan evolutionary time. Initial results from analysis family#3 suggests that these proteins are distant adaptations of a family#1 receptor. These analyses are performed on amino acid sequences to allow correlative structure/function analyses of mutant receptors to placed in an evolutionary perspective, as parsimony methods allow the tracing of amino acid positions through trees. In addition to the details of the evolution of these receptors investigated using these methods, the most applicable finding from this work is that we can classify newly discovered orphan receptors. for which such biological information as its native ligand and second messenger signaling systems are unknown. The relationships of these receptors to other better characterized receptors provides a rational basis for the analysis of the biological function of these poorly characterized proteins.

I 144 PROTEIN KINASE C LOCALIZATION AND PHOSPHATIDYLCHOLINE HYDROLYSIS IS STIMULATED IN THE NUCLEUS OF α-THROMBIN-TREATED IIC9 CELLS. Karen L. Leach, Valerie A. Ruff, Matt B. Jarpe, and Daniel M. Raben Department of Cell Biology, The Upjohn Company, and Department of Physiology, Johns Hopkins University School of Medicine.

An understanding of how signals originating at the plasma membrane are communicated to the nucleus is an important biological question. We have previously shown (*J. Biol. Chem.* **266**, 3215) that α-thrombin treatment of IIC9 cells results in increased levels of cellular 1,2-diacylglycerol (DAG) and activation of protein kinase C (PKC). We show here that changes in nuclear PKC and nuclear DAG are also induced following α-thrombin treatment. IIC9 cells were treated with 500 ng/ml α-thrombin and nuclei were then isolated. PKC α, but not PKC ε or ζ, was present in the nuclei of cells treated with *either phorbol* 12,13-myristate (PMA) or α-thrombin. The rise in nuclear PKC levels occurred rapidly and reached a maximum at 30-60 sec, which was followed by a decline back to the control level over the next 15 min. In addition, α-thrombin treatment resulted in an immediate rise in DAG mass levels in the nuclear fractions. The potential source of the induced nuclear diglycerides was identified by carrying out molecular species profiles of both the induced diglycerides and nuclear phospholipids by capillary gas chromatography. The molecular species profiles of the nuclear diglycerides genereated resembled the species profiles of phosphatidylcholine, and not phosphoinositides species, at all times. Our results suggest that increases in both nuclear DAG levels and PKC activity following α-thrombin treatment may play a role in mediating thrombin-induced nuclear responses such as changes in gene expression and cellular proliferation.

I 145 INVOLVEMENT OF A CALMODULIN-DEPENDENT PROTEIN KINASE IN LIGHT-REGULATED ROOT GRAVITROPISM IN MAIZE, Ying-Tang Lu and Lewis J. Feldman, Department of Plant Biology, University of California, Berkeley, CA 94720

The gravity response (gravitropism) in roots of many plants is triggered by and is dependent on light which affects signal transduction processes in the root cap. Calcium is known to be involved with light-regulated gravitropism, but the mechanism of action is not known. Recently, a role for protein phosphorylation has been suggested. Using the drug KN-93 which specifically and competitively inhibits calmodulin (CaM) binding to the CaM-binding domain of a calmodulin-dependent protein kinase (CaMK), we have demonstrated that CaMK may be involved in mediating light-regulated gravitropism in roots of maize. KN-93 blocks light-induced gravitropism but does not inhibit either the perception of light or root growth. A cDNA clone (1.9 kb) encoding a CaMK has been isolated and characterized from maize (*Zea mays*, var. Merit). It is expressed in root tips, and especially in the root cap. The encoded peptide contains a protein kinase catalytic domain with all 11 subdomains and conserved amino acid residues. Using peptide analysis, its CaM-binding domain is located at the same location as in yeast and in mammalian CaMK II. Supporting evidence comes from ^{35}S-CaM gel overlay analysis of the fusion protein showing that CaM binding is calcium-dependent. CaM binding is inhibited by 50 μM KN-93, while not affected by 5 μM KN-93. This drug is very specific for our CaMK as evidenced by the fact that it does not affect CaM-binding to other proteins in the maize root tip, at either 0, 5 or 50 μM KN-93. These results agree with data that the gravitropic response of Merit roots is inhibited by 50 μM KN-93 but not by 5 μM KN-93, supporting the view that CaMK plays a role in light-regulated root gravitropism.

I 146 EVIDENCE OF A RYANODINE-SENSITIVE CALCIUM RELEASE CHANNEL IN CULTURED HUMAN MYOMETRIAL SMOOTH MUSCLE CELLS, Stephen Lynn, Joanna M. Morgan, James I. Gillespie & John R. Greenwell, Department of Physiological Sciences, The Medical School, University of Newcastle upon Tyne, NE2 4HH. U.K.

Single cultured human myometrial cells demonstrate a large release of intracellular calcium, which is derived from an internal store, in response to brief applications of the plant alkaloid, ryanodine (1μM). It is possible to induce oscillations in intracellular calcium in these cells by the continuous application of the hormone, oxytocin (1nM). These oxytocin-induced oscillations, which involve the production of inositol trisphosphate (IP_3), are reversibly inhibited by ryanodine (1μM). This may suggest that ryanodine exerts it's effect on the IP_3-sensitive calcium store. Ryanodine was also observed to mobilise intracellular calcium in cells bathed in a nominally calcium-free solution (<1μM Ca^{2+}), further implying that an internal store is sensitive to ryanodine.

The application of caffeine (10mM) was found to reversibly inhibit the oxytocin-induced oscillations in these cells, possibly as a consequence of it's ability to interfere with the production of IP_3. However, application of caffeine was found not to mobilise intracellular calcium in non-stimulated cells, but a small decrease in the resting calcium level was observed, which may be due to the inhibition of receptor-activated calcium influx. These data also show that the lack of calcium mobilisation in response to caffeine indicates that these cells do not possess the classical caffeine/ryanodine-sensitive intracellular calcium store found in cardiac muscle.

It is possible that both ryanodine and IP_3 receptors may be co-localised on the same internal store. The binding of ryanodine to these receptors may lock the channel in an open, low conductance state and deplete the store which is slowly refilled when ryanodine is removed.

This suggests evidence of a novel caffeine-insensitive ryanodine-sensitive calcium release mechanism in these cells, which may be similar to that described in other smooth muscle cells and areas of the brain. This may be the first evidence of a type 3 ryanodine receptor (RYR-3) in these cells to be reported.

I 147 **CLONING OF A NEW β-TYPE PHOSPHOLIPASE C FROM *XENOPUS* OOCYTE AND CHARACTERIZATION OF ITS G PROTEIN-DEPENDENT REGULATION**, Hai-Wen Ma, Robert D. Blitzer, Emmanuel M. Landau, and Ravi Iyengar, Departments of Pharmacology, Molecular Biology and Psychiatry, Mount Sinai School of Medicine, CUNY, New York, NY 10029

Xenopus oocytes exhibit a receptor-evoked Cl^- current that is mediated through the activation of phospholipase C (PLC) and the subsequent increase in $[Ca^{2+}]_i$. Endogenous angiotensin receptors and many exogenous receptors expressed in ooctyes couple to this pathway, at least partially through pertussis toxin sensitive G protein(s). We have cloned cDNAs encoding a new form of PLC (PLC-*X*β) from a *Xenopus* oocyte cDNA library. These cDNAs encode a protein 1210 amino acids long. PLC-*X*β is most similar in sequence and overall structure to the β-class of mammalian PLCs. Injection into oocytes of antisense, but not sense, oligonucleotides to a specific region of PLC-*X*β results in degradation of its mRNA and a significant reduction of Cl^- currents evoked by both endogenous angiotensin receptors, as well as expressed mammalian α_{1b}-adrenergic receptors and M_1-muscarinic receptors. Inhibition of the M_1-muscarinic response produced by antisense oligonucleotides was nonadditive with that produced by pertussis toxin. These results indicate that PLC-*X*β is involved in the pertussis toxin senstive receptor stimulation of the Ca^{2+} activated Cl^- current in *Xenopus* oocytes. The full length PLC-*X*β cDNA has been reconstructed and inserted into a mammalian expression vector. Studies are underway to characterize the regulation of PLC-*X*β by various G protein subunits. Results from these studies will be presented.

I 148 SPHINGOMYELINASE ACTIVATES THE PROTEOLYTIC IKB DEGRADATION PATHWAY IN A CELL-FREE SYSTEM, Thomas Machleidt*, Katja Wiegmann*, Thomas Henkel[§], Stefan Schütze*, Patrick Baeuerle[§], and Martin Krönke*[§] *Institut für Mikrobiologie und Hygiene, Trogerstr. 4A, Technische Universität, 81675 München, Germany and [§]Laboratory for Molecular Biology, Gene Center, Am Klopferspitz 18a, Martinsried, Germany

Tumor necrosis factor is one of the most potent physiological inducers of the nuclear transcription factor κB. A key event in the activation of NF-κB is the rapid release of the inhibitory subunit IκB. In this study various inhibitors of serine-like proteases are shown to block not only TNF-mediated NF-κB activation but also disappearance of IκB-α immunoreactivity in primary murine spleen T lymphocytes as well as in various human leukemic cell lines. The protease inhibitors used did not block TNF-induced activation of either phosphatidylcholine -specific phospholipase C (PC-PLC) or acidic sphingomyelinase (SMase), indicating that the putative protease does not interfere with proximal TNF signal transduction processes. Strikingly, IκB-α degradation can be directly induced by exogenous sphingomyelinase in a cell-free system, indicating a stringent coupling of SMase to the NF-κB activation pathway. SMase-induced IκB degradation could be prevented by the protease inhibitor dichloroisocoumarin, suggesting that a TNF-responsive sphingomyelinase triggers the rapid degradation of IκB-α through a serine-like protease, which appears to be crucial to the control of NF-κB activation.

I 149 ROD TRANSDUCIN IS PRESENT IN TASTE CELLS. **Robert F. Margolskee, Susan K. McLaughlin, Peter J. McKinnon, Alain Robichon and Nancy Spickofsky, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.**

The guanine nucleotide binding proteins (G proteins) mediate signal transduction in olfactory, visual, and gustatory systems. The G proteins comprise a family of proteins which transduce an extracellular signal into an intracellular second messenger (e.g. cAMP, cGMP, IP3). In the vertebrate taste cell, G proteins are involved in the transduction of both bitter and sweet tastants. Some bitter compounds raise the intracellular calcium concentration in rat taste cells, apparently via G protein induced IP3. Sucrose causes a G protein-dependent generation of cAMP in rat taste cells which in turn depolarizes the cell. To identify and characterize those proteins involved in the taste transduction process, we have cloned G protein α subunit cDNAs from a rat taste cell cDNA library. To date, eight distinct α subunit cDNAs have been isolated, cloned and sequenced from this library. We previously showed that α gustducin is taste cell specific and very closely related to the transducins (the photoreceptor G proteins). We have also cloned the α subunit of rod transducin from taste cells. Transducin mRNA and protein are expressed in taste tissue and retina, but absent from control tissues. *In situ* hybridization and immunohistochemistry demonstrate that transducin is specifically expressed in the taste receptor cells. Presumably, both transducin and gustducin are involved in taste transduction.

I 150 TRANSDUCIN ACTIVATION AND INACTIVATION: OPSIN, GTP ANALOGUES, AND GTPASE ACCELERATION, Thomas J. Melia, Joseph K. Angleson, Evelyn Zera, Justine A. Malinski, David P, Molloy, Jagannath B. Lamture, and Theodore G. Wensel, Verna & Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030

Recent studies of the activation/inactivation cycle for the visual G protein transducin (G_t) have revealed several surprising features: 1) Opsin (*i.e.*, ligand-free receptor) appears to be capable of catalyzing $G_{t\alpha}$ activation at detectable rates; these are orders of magnitude slower than R* (opsin with the agonist all-*trans* retinal bound) but significantly faster than estimated for rhodopsin (opsin with the antagonist 11-*cis* retinal bound). 2) Binding of $G_{t\alpha}$ to GTP or GTPγS is much tighter than previously thought (K_d ~ 100 pM), but the binding of GDPβS or ribose-modified GTP analogues (*e.g.* 2'-/3'- (N-methylanthraniloyl)-GTP, or mant-GTP) is orders of magnitude weaker. GDPβS does not inhibit $G_{t\alpha}$ activation, and activation of $G_{t\alpha}$ by mant-GTP is too weak to be reliably detected. 3) One or more protein factors in rod outer segment membranes distinct from cGMP phosphodiesterase subunits can both accelerate $G_{t\alpha}$ GTPase and enhance acceleration of $G_{t\alpha}$ GTPase by the inhibitory γ subunit of the phosphodiesterase. Supported by NIH Grants EY07981, EY07001, & GM08280, and by the Welch Foundation.

I 151 PKCθ IS EXPRESSED IN HUMAN HEAMTOPOIETIC CELLS, REGULATED BY PHORBOL ESTERS AND EXHIBITS A UNIQUE SUBSTRATE SPECIFICITY. N.Meller*, G. Baier# ,D. Galron*, A. Tamir*, D. Telford#, L. Giampa#, K.M. Coggeshall# ,G. Baier-Bitterlich#, A. Altman# and N. Isakov*.*Dept. Microbiol. & immunol. & Cancer Res. Ctr., Ben Gurion Univ., Beer Sheva, Israel & #La Jolla Inst. Allergy & Immunol., La Jolla, CA, USA.

Protein kinase C (**PKC**) θ is a new member of the PKC family that was recently cloned from human leukemic T cell line (Jurkat) derived cDNA library. PKCθ contains a consensus protein kinase catalytic domain and a phospholipid and diacylglycerol binding domain but lacks a Ca^{2+} binding domain. It exhibits the highest homology with PKCδ and possesses a unique sequence in its V3 domain. Using rabbit antiserum directed againsed a bacterially expressed V3 domain of PKCθ we immunoprecipitated a 82 kDa protein from the human leukemic T cell line, Jurkat, that reacted in immunoblot with anti-PKCθ, but not with preimmune serum. Western blot analysis demonstrated PKCθ expression in human T cell lines (Jurkat, CEM-K, HPB.MLT, HUT78) and histiocytic cell line, U937, but not with normal epidermal keratinocytes, a melanoma cell line (A375), colon carcinoma lines (HT-29-N2, SW620), a human fibroblastoid cell line (233) B cell line (Raji) or a shwan cells (TC620).PKCθ responded by cytosol-to-membrane translocation upon stimulation of T cells with a phorbol ester and undergone an enhanced degradation in the presence of the PKC activator, bryostatin. we have transfected COS1 cells with a plasmid encoding PKCθ plus 6 tandem histidine residues in its carboxy terminal and used a Ni^{2+}-resin column to purify the enzyme. Preliminary results indicated that PKCθ phosphorylates a synthetic peptide corresponding to the PKC-pseudosubstrate prototype in which Ala^{25} was substituted with Ser , but not other PKC substrates such as histone H1, myelin basic protein synthetic peptide or protamin.

I 152 OXYTOCIN-INDUCED OSCILLATIONS IN INTRACELLULAR CALCIUM IN DEPOLARISED CULTURED HUMAN MYOMETRIAL SMOOTH MUSCLE CELLS, Joanna M. Morgan, Stephen Lynn, James I. Gillespie and John R. Greenwell, Department of Physiological Sciences, The Medical School, University of Newcastle upon Tyne, NE2 4HH. U.K.

Spontaneous and agonist-induced oscillations in intracellular calcium have been recorded in cultured human myometrial smooth muscle cells. These oscillations persist in solutions containing high concentrations of potassium (140mM) suggesting that the underlying mechanism does not involve the cyclical operation of voltage-dependant ion channels. Intermediate increases in external potassium (10-70mM) can lead to an increase in the frequency of the calcium oscillations suggesting that membrane potential regulated mechanisms do operate in these cells but that their function may be primarily to modulate the frequency of the calcium oscillations.

The hypothesis is proposed that repetitive calcium spikes involve the cyclical release of calcium from an internal store, possibly a single calcium store, sensitive to inositol trisphosphate (IP_3) and activated by intracellular calcium. As a result of membrane depolarisation an increased calcium influx could modulate the frequency of the calcium spikes by influencing basal levels of cytoplasmic calcium. This cyclical release may be modulated by elevating IP_3 (via agonist stimulation) which functions *effectively as a modulator* rather than a primary intracellular signal to alter the sensitivity of the calcium release process to calcium.

In human labour the plasma concentration of oxytocin and prostaglandins do not change in the period leading up to contractions and do not fluctuate during the early phases of labour. Therefore, it is not clear what contribution these hormones play in the phasic activity of the human myometrium. However, if the role of such hormones was to raise IP_3 to modulate the cyclical release of calcium from internal stores, possibly triggered or augmented by a calcium influx via voltage gated calcium channels, the myometrium would function in a phasic manner in the presence of a steady background level of hormone.

I 153 **AUTOCRINE STIMULATION OF YEAST THROUGH HUMAN G-COUPLED RECEPTORS,** Murphy, A.J.M.; Paul J.; Manfredi, J.; Silverman, L.; Trueheart, J.; McKinney, M. and Broach, J. Cadus Pharmaceutical Corporation, 180 Varick St., N.Y., N.Y.

Response to mating factors in the yeast *Saccharomyces cerevisiae* is coupled to transcription through a seven transmembrane receptor, a heterotrimeric G-protein and a series of protein kinases. By incorporating a pheromone responsive selectable marker construct (fus1-HIS3), we have generated a growth read-out for activation of the pathway. Through a series of additional modifications, including the replacement of the yeast Gα subunit (GPA1) with a modified human $G\alpha_{i2}$, we have functionally substituted the yeast pheromone receptor with the human angiotensin II-type I receptor. When expressed in yeast, this receptor binds ligands with appropriate specificity and affinity. Co-expression of the angiotensin II octapeptide generates an autocrine loop in which the endogenously produced peptide stimulates the co-expressed receptor. This yields prototrophic growth dependent on expression of both angiotensin receptor and the angiotensin II peptide. This system provides a facile microbiological assay for angiotensin II variants, receptor mutants and external agents that affect signal transduction through this mammalian G-coupled receptor.

I 154 A DOMINANT NEGATIVE MUTANT OF G_{i2} INHIBITS PHOSPHOLIPASE A_2 STIMULATION IN CHO CELLS. Rosemary Murray-Whelan, John Reid*, Isabelle Piuz and Werner Schlegel, Fondation pour Recherches Medicales, University of Geneva, 64 Av de la Roseraie, 1211 Geneva 4 and *Glaxo IMB, 64 Chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland.

The specificity of interaction of receptors, G proteins and effectors is a central question in cell regulation. A powerful approach to this question is the use of mutated G proteins which either interrupt or constitutively activate the signalling pathway. We have generated a dominant inhibitor of G_{i2} which demonstrates the role of G_{i2} in PLA_2 activation in CHO cells. A mutant form of α_{i2} in which glycine 203 was converted to threonine (G203T) was made by site-directed mutagenesis. When expressed in CHO cells, G203T demonstrates a dominant negative phenotype. G203T had no effect on basal levels of arachidonic acid release. However, both thrombin and ATP stimulation of PLA_2 were 50-60% inhibited by G203T. In contrast, stimulation of calcium release by either thrombin or ATP, which is pertussis toxin insensitive, was not affected by the mutant. The effect of the mutant is thus specific for G_{i2} mediated signal transduction. The *gip* oncogene (α_{i2} Q205L mutant) is a constitutively active mutant of G_{i2} which has previously been shown to inhibit both adenylate cyclase and PLA_2 in CHO cells. Unlike Q205L, however, G203T had no effect on stimulation of adenylyl cyclase activity by either cholera toxin or forskolin. In addition, receptor-independent activation of arachidonic acid release by PMA was unaffected by G203T, while PLA_2 activation by PMA is decreased by 20-25% in Q205L-expressing cells. These data indicate that the inhibitory effect of G203T on thrombin and ATP stimulation of PLA_2 is mediated directly at the G protein level, while Q205L inhibition is probably secondary to activation and subsequent desensitization. Thus mutation of glycine 203 of the α_{i2} protein inactivates G_{i2} and produces a dominant negative inhibitor of endogenous G_{i2} activation of PLA_2.

I 155 CALCIUM RESPONSES TO TRH: AGONIST-STIMULATED EFFLUX OF CYTOPLASMIC CALCIUM. Eric J. Nelson and Patricia M. Hinkle. Dept. of Pharmacology, Univ. of Rochester Med. Sch., Rochester, NY 14642

TRH is a calcium-mobilizing hormone that stimulates immediate release of Ca^{2+} from intracellular, IP_3-sensitive stores. Using single cell imaging we have investigated the TRH response in Fura-2-loaded GH_3 pituitary cells expressing endogenous receptor and Hela cells transfected with the TRH receptor cDNA. The amplitude of the initial $[Ca^{2+}]i$ transient increased as the TRH concentration was raised. However, the duration of the initial $[Ca^{2+}]i$ transient decreased at high TRH concentrations, suggesting that TRH was stimulating both increased release of Ca^{2+} from the endoplasmic reticulum via IP_3 and increased clearing of Ca^{2+} from the cytoplasm. TRH caused $[Ca^{2+}]i$ to go down when it was added to GH_3 cells after $[Ca^{2+}]i$ had been increased by: ionomycin, a calcium ionophore; high potassium, which depolarizes cells and increases influx through L-channels; or thapsigargin, which depletes IP_3-sensitive calcium stores. TRH-stimulated Ca^{2+} clearing could not be mimicked by phorbol esters, suggesting that protein kinase C activation is not sufficient to cause the effect. TRH-induced Ca^{2+} clearing was dose-dependent in both cell lines. Following depletion of IP_3-sensitive Ca^{2+} pools, TRH caused Ca^{2+} clearing without the TRH-evoked transient increase in $[Ca^{2+}]i$. TRH-stimulated Ca^{2+} clearing did not result from an effect on calcium channels because: TRH increased Ca^{2+} clearing in transfected Hela cells, which lack calcium channels; in GH_3 cells, TRH increased Ca^{2+} clearing while L-type calcium channels were blocked; finally, TRH increased Ca^{2+} clearing when GH_3 cells were incubated in EGTA to chelate extracellular Ca^{2+} or in medium containing a 100-fold range of Ca^{2+} concentrations. These results show that TRH causes $[Ca^{2+}]i$ to fall by stimulating Ca^{2+} efflux. Complete replacement of extracellular sodium did not inhibit TRH-stimulated Ca^{2+} clearing, so Ca^{2+} efflux via the sodium-calcium exchanger is not involved. The results suggest that calcium-mobilizing agonists may activate a plasma membrane calcium ATPase. In summary, TRH initiates both a $[Ca^{2+}]i$ transient and agonist-activated clearing of cytosolic Ca^{2+}, which prevents prolonged elevation of $[Ca^{2+}]i$ and terminates the $[Ca^{2+}]i$ signal.

I 156 TGF-ß MEDIATES THE PRODUCTION OF 1,2-DIACYLGLYCEROL AND CHOLINE PHOSPHATE IN A HAEMATOPOIETIC PROGENITOR CELL LINE.

Anne-Marie O'Farrell*#, A.D. Whetton%, P. Jane Owen Lynch%, T. Michael Dexter# and Clare M. Heyworth#. # Christie Hospital NHS Trust, Withington, Manchester M20 9BX, UK. % Leukaemia Research Fund Group, Dept. UMIST, Sackville St., Manchester M60 1QD, UK. *current address; DNAX Research Institute, 901 California Ave., Palo Alto, CA 94304, USA.

The haemopoietic cell line Cl 15G is dependent on either interleukin-3 (IL-3) or granulocyte colony-stimulating factor (G-CSF) for survival and proliferation *in vitro*. When TGF-ß was added to Cl 15G cells, it inhibited the G-CSF or IL-3-stimulated proliferation of Cl 15G cells in a time and dose-dependent manner. TGF-ß-mediated inhibition of G-CSF stimulated proliferation was dependent upon the concentration of G-CSF present. There was however no TGF-ß-stimulated down-modulation of G-CSF receptors after 2 or 48 hours of treatment with TGF-ß. Thus the antiproliferative effects of TGF-ß on G-CSF-stimulated proliferation would appear to occur at a level distal from the modulation of growth factor receptors.

Addition of TGF-ß to Cl 15G cells stimulated an increase in the level of *sn*1,2-diacylglycerol (DAG) and phosphatidylcholine (PtdCho) breakdown to produce choline phosphate (PCh) in Cl 15G cells. Thus the stimulation of PtdCho hydrolysis by phospholipase C may be a mechanism of TGF-ß action unique to haemopoietic progenitor cells. Neither G-CSF, IL-3 nor TGF-ß significantly stimulated phosphatidylinositol breakdown or increased cAMP production in Cl 15G cells. However G-CSF stimulated a dose-dependent activation of the Na^+/H^+ antiport which was reduced by the addition of TGF-ß.

The Cl 15G cell line provides preliminary evidence of biochemical events which may be involved in mediating the effects of TGF-ß in haematopoietic progenitor cells, and provides a powerful model for further examination of the modes of action of haematopoietic growth regulators.

I 157 THE AUTOINHIBITORY DOMAIN OF CALCINEURIN REGULATES PHOSPHATASE ACTIVITY AND INTERACTION WITH THE FK-506•FKBP12 COMPLEX, Stephen J. O'Keefe*, Randall L. Kincaid†, Gregory J. Wiederrecht‡, Jonathan J. Burbaum§, Laura L. Rokosz§ and Janey N. Parsons*; *Dept. of Molecular Immunology, ‡Dept. of Cellular Immunology and §Dept. of Biophysical Chemistry Merck Research Labs, Rahway, NJ 07065 and †Immunology Sect., NIAAA/NIH, Rockville, MD 20852.

FK-506 is a clinically relevant immunosuppresive drug that forms a complex with a ubiquitous intracellular binding protein, FKBP12. The FK-506•FKBP12 complex inhibits the heterodimeric Ca^{+2}/calmodulin-dependent phosphatase, calcineurin (CaN), an essential component of the T cell receptor signal transduction pathway. Because the drug•binding protein complex inhibits CaNs from such diverse species as yeast and man, its binding site must be highly conserved. Identification and characterization of the drug•BP binding site might lead to the design of new immunosuppressants with an increased therapeutic index. As a first step we have begun to define regions within CaN, such as the conserved autoinhibitory domain, that are responsible for regulating phosphatase activity and the interaction with the FK-506•FKBP complex. Deletion of the nonconserved carboxy terminus of the C subunit (ΔCT) had no effect on the normal regulation of phosphatase activity compared to the wild type enzyme, based on an *in vivo* assay. Deletion of the putative autoinhibitory domain (ΔAI), however, generated a Ca^{+2}-independent enzyme, confirming the role of this region in regulating enzyme activity. To assess the role of the autoinhibitory domain in regulating the interaction of CaN with the FK-506•FKBP12 complex, we reconstituted the holoenzyme using subunits produced by *in vitro* transcription/translation. Binding of the wild type enzyme to the FK-506•FKBP12 complex, as well as that of the truncated enzymes, was completely dependent on the presence of FK-506. Association of the wild type enzyme and ΔCT were completely dependent upon Ca^{+2} since the addition of 10 mM EGTA eliminated complex formation. Binding of the Ca^{+2}-independent ΔAI, however, was unaffected by EGTA. These results indicate that the conserved autoinhibitory domain prevents association of CaN with FK-506•FKBP12 and suggest that the binding sites of the FK-506•FKBP12 complex and the autoinhibitory domain overlap.

I 158 REGION OF RAF-1 PROTEIN KINASE RESPONSIBLE FOR THE SERUM- AND PHORBOL ESTER-INDUCED SHIFT IN GEL MOBILITY APPEARS TO LOCALIZE WITHIN THE 33 kDa C TERMINAL DOMAIN, Zoltan Olah, Alma Ferrier, Csaba Lehel and Wayne B. Anderson, Laboratory of Cellular Oncology, National Cancer Institute, NIH Bethesda, MD 20892

Experiments were designed to identify the Raf-1 protein kinase domain responsible for the upshift in SDS-PAGE gel mobility, noted with serum and phorbol ester (PMA) treatment of serum-deprived NIH 3T3 cells. A new metallothionein promoter driven ε-epitope tagging vector, the εMTH was used to overexpress ε-tagged holo Raf-1 (HR-ε) as well as three ε-tagged Raf-1 fragments designed to cover the entire length of Raf-1. The recombinant proteins overexpressed in NIH 3T3 cells each contain the ε-tag peptide at the C-terminus to allow ready detection with an antibody specific for the ε peptide. Transfection with these vectors resulted in the Zn^{2+}-inducible overexpression of 84 kDa HR-ε, 35 kDa N-terminal fragment (RI-ε), and 33 kDa C-terminal fragment (RIII-ε). The ε-tagged protein corresponding to the 33 kDa middle fragment (RII-ε) of Raf-1 appeared to be unstable, and little RII-ε protein accumulated in the RII-ε transfected cells. Results indicated that C-terminal ε-tagging did not interfere with biological activity. The introduced HR-ε exhibited a serum- and PMA induced shift in gel mobility similar to that noted for endogenous Raf-1. The C-terminal RIII-ε fragment showed a similar shift in gel mobility, while the mobility of the N-terminal RI-ε fragment remained unchanged. [^{32}P] Phosphate incorporation studies, and experiments with various ser/thr and tyr phosphoprotein phosphatases, indicated that phosphorylation may not fully account for the upshift of Raf-1 gel mobility. These results suggest that modification(s) within the 33 kDa C-terminal portion of Raf-1 may be responsible for the band shift observed with serum and PMA treatment of serum-deprived NIH 3T3 cells.

I 159 IDENTIFICATION OF HUMAN cDNA CLONES ENCODING POTENTIAL NOVEL PKC RELATED KINASES

Ruth H. Palmer, John Ridden and Peter J. Parker, Imperial Cancer Research Fund, P.O.Box 123, 44, Lincoln's Inn Fields, London, WC2A 3PX

Protein Kinase C (PKC) comprises a large and growing multigene family of serine/threonine protein kinases. In order to further investigate the heterogeneity within the PKC family we have employed PCR using degenerate oligonucleotides based on a region conserved over all PKC isotypes. Analysis of the resulting PCR products revealed that it contained existing PKC as well as potential novel kinase sequences that may define a distinct gene family. Several of the subcloned PCR fragments have been used to screen human cDNA libraries. This has resulted in the identification of several cDNA clones.

Partial and full length clones have been isolated for two members of this PKC-related novel gene family. Patterns of expression, chromosomal localisation and the nature of the encoded proteins are currently under investigation.

I 160 SIGNAL TRANSDUCTION PATHWAYS ALTERED BY A CONSTITUTIVE INCREASE IN INTERNAL pH. Rosario Perona, José R. Murguía and L. Gomez-García.Instituto de Investigaciones Biomédicas del CSIC, C/Arturo Duperier,4. Madrid.

The role of pH^{in} the regulation of cell proliferation of both normal and transformed cells is a topic of considerably controversy. We have transfected NIH3T3 cells with the gene of a yeast proton pumping ATPase PMA-1. These cells are tumorigenic in nude mice and are able to grow in the absence of serum. The pH^{in} is 0.3 units more alkaline than that of the parental ones even in the presence of bicarbonate. By using site directed mutants it has been demostrated that the degree of transformation, tumorigenicity and pH^{in} directly correlates with the activity of the enzyme. The expression of the PMA-1 gene is able to transactivate the c-fos promoter and increase levels of AP-1 transcription factor independently of protein kinase C activity. Stable transfectants of the yeast proton pump (RN1a) have higher cytoplasmic calcium concentration mediated by calcium channels. The high constitutive levels of AP-1 transcription factor and serum independent growth are dependent on high intracellular calcium levels. Furthermore inhibition of phospholipase A_2 and the 5-lipoxygenase activities causes a decrease in AP-1 complex levels without affecting calcium uptake. The growth factor response is altered also by modulation of cytoplasmic calcium. RN1a cells show similar increase than NIH3T3 cells in response to PDGF-BB and EGF but they are non responsive to PDGF-AA and grew in the presence of IGF-1 and insulin at nM concentrations. An artificial decrease in the levels of intracellular calcium makes the RN1a cells responsive to PDGF-AA without affecting the responses to the other growth factors. The addition of calcium back to the media abolishes the response to PDGF-AA. Constitutive tyrosine kinase activity of the RN1a cells is also dependent on calcium concentration. These results suggest that a constitutive alkalinization of pH^{in} causes and increase of intracellular calcium levels, in tyrosine kinase activity and in the metabolites of araquidonic acid. The activation of these transduction pathways is involved in the activation of AP-1 and low serum growth of the transformed cells.

I 161 **KINETICS OF GUANINE NUCLEOTIDE BINDING AND HYDROLYSIS AND G PROTEIN ACTIVATION MEASURED BY FLUORESCENT GUANINE NUCLEOTIDE ANALOGS.** Ann E. Remmers & Richard R. Neubig, Department of Pharmacology, The University of Michigan, Ann Arbor, MI 48109.

The N-methyl-3'-*o*-anthranoyl (MANT) guanine nucleotide analogs are useful environmentally sensitive fluorescent probes to study G protein mechanisms. Upon binding to G_o, both MANT-GTPγS and MANT-GTP display increased fluorescence. The increase in MANT-GTP fluorescence was greater with excitation at 280 nm which excites the MANT via energy transfer from G_o compared to excitation of the MANT directly at 350 nm. MANT-GTPγS fluorescence increases exponentially with time and the rate of the increase is identical to the rate of G_o tryptophan quenching by MANT-GTPγS suggesting that resonance energy transfer between G_o and MANT is occurring. The increase in MANT-GTP fluorescence is followed by a decrease in fluorescence as MANT-GTP is hydrolyzed to MANT-GDP. The rates of MANT-GTPγS binding to G_o were slow. Mastoparan increased the rates with an EC_{50} of 54 ± 0.5 μM. Kinetic modelling provides evidence that mastoparan speeds the conformational change associated with Go activation. Two components were observed in the dissociation kinetics of MANT-GTP from G_o. The rate constants were 0.414 ± 0.008 sec^{-1} and 0.0275 ± 0.0009 sec^{-1}. The amplitude of the rapidly dissociating component increases over time and appears to represent MANT-GDP dissociation. The amplitude of the slowly dissociating component increases and then decreases representing the binding and hydrolysis of MANT-GTP. The rate of the slow component would be governed by the sum of the rates of MANT-GTP dissociation from G_o and MANT-GTP hydrolysis. Unlike ras, when bound to G_o, the MANT-guanine nucleotide emission spectrum is shifted to the blue by 13 nm indicating a nonpolar environment for the bound MANT nucleotide. The fluorescence changes in MANT-GTP upon binding G_o are useful indicators of kinetic rates of both nucleotide binding and release as well as G protein activation and inactivation. (Supported by NIH GM 39561 and GM 14654.)

I 162 FUNCTIONAL CHARACTERIZATION OF THE SIGNAL TRANSDUCTION EVENTS MEDIATED BY FcεRI *α* AND *γ* CHIMERIC RECEPTORS, Barbara Repetto, Geethani Bandara, Helen Kado-Fong, Douglas Larigan, Gloria A. Wiggan, Alasdair M. Gilfillan and Jarema P. Kochan. Bronchopulmonary Department, Hoffmann La-Roche, Nutley, N.J. 07110

To characterize the functional contributions of the FcεRI *α* and *γ* subunit domains during signal transduction, we stably transfected chimeric receptors into RBL-2H3 cells. The extracellular (EC) domain of the human *α* subunit was fused to the human *γ* subunit transmembrane (TM) and cytoplasmic (CT) domains, generating the chimera *α/γ/γ*. Characterization of stable RBL clones expressing this chimera demonstrated that it forms a fully functional complex with the endogenous rat *β* and *γ* subunits and is capable of mediating the following events: tyrosine phosphorylation, inositol phosphate (IP) production, phosphatidic acid (PA), 1,2 diacylglycerol production (DAG), increase in $[Ca^{++}]_i$, and secretion ($[^{14}C]$-arachidonic acid metabolites and histamine release). A single chain receptor was generated by replacing the *γ* TM of *α/γ/γ* with the p55 TM of the Il-2 receptor (I) to form *α*/I/*γ*. This single chain chimera does not associate with the endogenous rat FcεRI subunits and is capable of mediating the biochemical events common to the native receptor. Chimeras containing the p55 EC domain, I/*γ*/*γ* and I/I/*γ* were similarly constructed and characterized. Crosslinking of I/*γ*/*γ* elicits responses similar to the native rat receptor. However, crosslinking of the single chain chimera, I/I/*γ* resulted in no secretion, although other monitored events were reduced. Another chimera, *α*/I/I in which the *γ* CT domain has been replaced with the CT of p55 does not associate with the endogenous rat subunits. Crosslinking of *α*/I/I produces only an initial increase in both PA and DAG in the absence of the other monitored signals. Our analysis of the signaling events mediated by different chimera demonstrates that both the *α* EC domain and the *γ* CT domain are key contributors to the signaling process in mast cells.

I 163 **DIFFERENTIAL REGULATION OF *FYN* AS COMPARED TO *FGR* PROTEINS BY MUTATIONS WITHIN THEIR NON-CATALYTIC DOMAINS.** Octavio Rivero, Keith Robbins. Laboratory of Cellular Development and Oncology. National Institute of Dental Research. Bethesda, MD 20892.

Members of the *src* family share a common primary structure, namely the presence of unique, SH3, SH2 and catalytic domains. Although aminoacid homology among members is very high, it is not clear whether their non-catalytic domains have identical functions. Mutations generated in non-catalytic domains of the *src* genes have effects that range from neutral to dramatic with respect to tyrosine kinase activity and transforming capability. Using *fyn* and *fgr* genes, as examples of *src* family kinases that are ubiquotously expressed and highly restricted, we have addressed the issue of non-catalytic element function. Each of the catalytic domains was deleted independently and point mutations in SH3 and SH2 domains were generated at residues that are conserved among family members. Mutations in *fyn* gene reproduced the effects observed previously for *src*. In contrast, effects for *fgr* mutations were distinct, suggesting differential regulation by non-catalytic elements of *fyn* as compared to *fgr*.

I 164 EXPRESSION OF CAR2, A PRESTALK-SPECIFIC CAMP RECEPTOR IN DICTYOSTELIUM, Charles L. Saxe III, Yimin Yu and Andrea Bauman, Department of Anatomy and Cell Biology, Emory University School of Medicine, Atlanta, GA, 30322-3030

CAR2, the product of the *carB* gene, is a cell surface, cAMP receptor present during the latter stages of Dictyostelium development. By use of *carB/lacZ* gene fusion constructs, we have established the expression pattern for *carB*. Expression is first detected in randomly distributed cells at the tight aggregate stage. These cells subsequently arrive at the tip and represent the anterior-most region of the slug. Upon the completion of development, *carB*-expressing cells are present in the stalk and the top of the mature fruiting body. This pattern is reminiscent of the expression another gene, *ecmA*., which has previously been used to identify a subset of prestalk cells, the prestalk A cells. There are subtle differences, however, with *ecmA* being more widely expressed than *carB*. Double immunofluorescence confirms that there are two populations of *ecmA*-expressing cells: those that also express *carB* and those that do not. Thus, it appears that *carB* expression, and therefore CAR2 receptor expression is restricted to a subset of prestalk A cells during Dictyostelium development. In addition, by using homologous recombination-mediated gene disruption, we have created CAR2-null mutations. These *carB*$^-$ cells are capable of completing multicellular aggregate formation but cannot proceed any further in development. No tips are formed, no slugs are made and terminal cytodifferentiation is blocked. Thus, CAR2-mediated signaling appears to be critical for completion of normal Dictyostelium development. The *carB/lacZ* constructs have also allowed us to examine the fate of cells normally destined to express *carB*, in *carB*-disrupted strains. The initial expression of *carB* is unperturbed in *carB*$^-$ cells. The cells are then capable of collecting together within the aggregate though their position within the structure is highly variable. Synergy experiments between *carB*+ and *carB*$^-$ cells suggest that *carB* is not required for the normal movement of *carB*-containing (or pstA) cells to the tip of the aggregate. Likewise, a number of the cAMP receptor-mediated changes in gene expression that occur during multicellular aggregation are normal in *carB*$^-$ cells. The basis for the profound effect on development of the lose of CAR2 is under further investigation. We have also begun experiments designed to rescue the *carB*$^-$ phenotype by overexpressing wild type, mutant or chimeric forms of CAR2. Progress will be presented.

I 165 A CONSTITUTIVELY ACTIVATING MUTATION OF THE LUTEINIZING HORMONE RECEPTOR (LHR) IN FAMILIAL MALE PRECOCIOUS PUBERTY (FMPP), Andrew Shenker, Louisa Laue, Shinji Kosugi, John J. Merendino, Jr., Takashi Minegishi, and Gordon B. Cutler, Jr., NIDDK and NICHD, National Institutes of Health, Bethesda, MD 20892, USA, Kyoto University School of Medicine, and Gunma University School of Medicine, Japan

FMPP is a gonadotropin-independent disorder that is inherited in an autosomal dominant, male-limited pattern. Affected males generally exhibit signs of puberty by age four. Testosterone production and Leydig cell hyperplasia occur in the context of prepubertal levels of LH. The LHR is a member of the family of G protein-coupled receptors, and we hypothesized that FMPP might be due to a mutant receptor that is activated in the presence of little or no agonist. DNA encoding residues 441 to 594 of the LHR was amplified with PCR and temperature gradient gel electrophoresis was used to screen for heterozygous mutations. A single A→G base change that results in substitution of Gly for Asp^{578} in the sixth transmembrane helix of the LHR was found in affected individuals from 8 different families. Linkage of the mutation to FMPP was indicated by *MspI* restriction digest analysis. We suspect that there is a common ancestral origin for the Asp^{578}→Gly mutation, but that different mutations may be found in other families with FMPP. COS-7 cells expressing mutant LHR exhibited markedly increased cAMP production in the absence of agonist (40% of maximal agonist-dependent stimulation), suggesting that autonomous Leydig cell activity in FMPP is caused by a constitutively activated receptor. The mutation has negligible effect on agonist affinity or on the ability of LHR to be maximally activated by agonist. Mutations of heptahelical receptors that mimic agonist occupancy can serve as a mechanism of human disease, and provide insight into the normal mechanism of receptor activation.

I 166 POINT MUTATIONS IN G PROTEIN β AND γ COILED-COIL DOMAINS ALTER DIMER ASSEMBLY, William F. Simonds, Regina M. Collins and Anja Garritsen*, Molecular Pathophysiology Branch, NIDDK, NIH, Bethesda, MD 20892 and *Organon Int., P.O. Box 20, 5340 BH Oss, The Netherlands

G protein β and γ subunits form a noncovalent heterodimer which functions with Gα to transduce signals from activated receptors to second-messenger pathways within the cell. The domains responsible for β and γ dimerization and for βγ complex interaction with Gα, receptor and effector molecules remain unknown. Recently a model was proposed for βγ complex formation involving formation of a coiled coil between α-helical regions in β and γ (Garritsen *et al*, PNAS 90, [1993], p. 7706). This model was supported by mutagenesis of the β subunit coiled-coil domain: an E10K point mutation, involving a heptad *g* position residue predicted to form an interhelical ionic bond with γ, blocked βγ dimer formation, as evidenced by failure of the β mutant to translocate to the cytosol upon transient cotransfection with a nonprenylated γ mutant, an effect characteristic of wild-type β. While an E10R β mutant, like E10K, fails to assemble with γ in the cotransfection assay, E10A shows an intermediate level of expression. Two heptad *b* position mutants, E12K and A26E, and one *c* position mutant, D27N, translocate normally with nonprenylated γ. These results are consistent with a coiled coil model of βγ interaction in which intersubunit association is governed primarily by interactions among residues in heptad positions *a*, *d*, *e* and *g* and mutations at positions *b*, *c* and *f* are well tolerated. Results of γ truncation and point mutation will also be presented. Definition of the structural requirements for βγ assembly provides a rational starting point for probing Gα, receptor and effector interaction domains of the heterodimer.

I 167 PHARMACOLOGICAL STUDIES OF THE EXAGGERATED CAPACITATIVE Ca^{2+} ENTRY MECHANISM IN SV40-TRANSFORMED SWISS 3T3 CELLS. Jesse E. Sisken, Terry G. Newcomb and Roland D. Mullins. Department of Microbiology and Immunology and Biomedical /Engineering Program, University of Kentucky, Lexington Ky. 40536

In many cell types, depletion of intracellular Ca^{2+} stores by various means is known to activate Ca^{2+} influx through plasma membrane Ca^{2+} channels but the mechanisms which regulate this influx and the channels involved are as yet unidentified. Using the Ca^{2+} sensitive photoprotein, aequorin, as our calcium probe, we previously showed that agonist-induced Ca^{2+} transients in SV40-transformed mouse 3T3 (SV3T3) cells are altered compared to those in untransformed 3T3 cells (Cell Calcium 14:539-549) and that the activity of the capacitative Ca^{2+} entry mechanism in response to thapsigargin (TG) is enhanced approximately 5-fold . Studies of this exaggerated response in SV3T3 cells indicates that it resembles capacitative influx systems seen in other cell types: it is insensitive to concentrations of verapamil or dihydropyridines known to affect L-type channels and it is blocked by membrane depolarization and, in part, by the tyrosine kinase inhibitor, genistein (see Lee et al, JBC 268:9945, 1993). However, we also find that the TG-induced influx is reduced by high concentrations of nifedipine (≥25uM) or verapamil (≥100uM) and is sensitive to retinoic acid, which has been reported to block T-type channels in a lymphocyte cell line (Bosma and Sidell J. Cell Physiol. 135:317, 1988) but not by phenytoin which can also block T-type Ca^{2+} channels (Twombly et al, J. Pharmacol. and Exptl. Therap. 246:189, 1988). Taken together, the available data indicate that capacitative Ca^{2+} influx occurs through a novel type of Ca^{2+} channel which is hyperactive in SV40-transformed 3T3 cells relative to 3T3 cells. The ability of retinoic acid to block this channel may be related to it's effects as an anti-proliferative and differentiation agent.

I 168 NUCLEAR CALCIUM SIGNALLING: IDENTIFICATION AND CHARACTERIZATION OF A NUCLEAR ISOFORM OF CaM KINASE. Srinivasan, M., Edman, C. and Schulman, H., Dept. of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA 94305-5332.

Multifunctional calcium/calmodulin-dependent protein kinase (CaM kinase) is a major intracellular mediator of calcium signaling. Although CaM kinase is implicated in regulating diverse calcium-mediated cellular events, it is not clear how CaM kinase achieves this in a specific and selective manner. Isoform specific intracellular targeting of CaM kinase could be one mechanism by which CaM kinase selectively transmits calcium signals to different cellular locations.

We have identified an isoform of CaM kinase (δ_B) which is targeted specifically to the nucleus. We have also identified and dissected the nuclear localization sequence (NLS) in this enzyme, by site directed mutagenesis. Furthermore, a chimeric construct of a cytoplasmic αCaM kinase and the NLS sequence from the δ_B isoform is targeted to the nucleus. Thus, δ_BCaM kinase is probably responsible for specifically mediating some of the calcium signals in the nucleus.

Cells typically express multiple CaM kinase isoforms which co-assemble into decameric holoenzymes. Interestingly, co-expressing the cytosolic isoform with a large excess of the nuclear isoform targets the entire holoenzyme to the nucleus. This suggests that CaM kinase enters the nucleus as a 500,000 dalton protein. Conversely, co-expressing the nuclear isoform with a large excess of the cytoplasmic isoform localizes the enzyme in the cytoplasm. This alternative targeting of the nuclear isoform could well be another mechanism for regulating CaM kinase's nuclear function.

I 169 THE VOLATILE ANESTHETIC HALOTHANE DEPLETES INTERNAL CA^{2+}-STORES WITHOUT INDUCING A CA^{2+}-INFLUX. Piet W.L.Tas, Helga Konrad and Karl Heinz Weis, Institute of Anesthesiology, University of Würzburg, 97080 Würzburg, Germany.

Agonists linked to inositol phospholipid hydrolysis, cause in addition to inositol 1,4,5 trisphosphate mediated Ca^{2+} release from internal stores, also the opening of cation channels permeable to Ca^{2+} in the cell membrane. Since many reports exist about the elevation of the cytoplasmic Ca^{2+} concentration of neural cells by general anesthetics, we studied whether these anesthetics deplete internal stores and if so, whether they also generate a Ca^{2+} influx.

The effect of anesthetics on internal Ca^{2+} stores was studied with rat glioma C6 cells in suspension culture loaded with the Ca^{2+}-indicator Fura-2/AM. Fluorescence was measured with an Aminco-Bowman spectrofotofluorimeter.

In nominally Ca^{2+}-free medium 4 mM halothane caused a slow depletion of Ca^{2+} from thapsigargin-sensitive Ca^{2+}-stores. Readdition of Ca^{2+} (final conc. 2 mM) to the extracellular medium however did not generate a Ca^{2+}-influx. Further experiments showed that halothane depressed the endothelin-1 induced Ca^{2+}-influx in a dose dependent manner (IC50 is ca 1 mM halothane). The absence of a Ca^{2+}-influx in the presence of halothane is therefore probably not due to a lack of activation, but most likely to an inhibitory effect on channel activation or conductance. Since the Ca^{2+}-indicator Quin-2 buffers the $Ca^{2+}{}_i$ in C6 glioma cells, we used quin-2 loaded C6 cells in order to study the effect of halothane on unidirectional Ca^{2+}-influx. Halothane inhibited the unidirectional influx with an IC50 of 2mM. Since the Ca^{2+} influx in Fura-2 loaded cells was inhibited stronger then in Quin-2 loaded cells, it seems likely that additional effects related to Ca^{2+}-signaling may play a role in the observed inhibition.

In conclusion: clinical concentrations (0.4 - 1.0 mM halothane) depress the endothelin-1 induced Ca^{2+}-entry into rat glioma C6 cells. If these channels are representative for those occurring in the central nervous system, it may be expected that halothane will depress the Ca^{2+}-signalling of those neurotransmitters which are linked to phospholipid hydrolysis.

I 170 ROLE OF βARRESTIN AND βARK IN MEDIATING THE HOMOLOGOUS DESENSITIZATION OF THE DOPAMINE D1A RECEPTOR. Mario Tiberi and Marc G. Caron. HHMI Labs. Department of Cell Biology, Duke University, Durham, NC 27710.

The D1A dopamine receptor subtype belongs to the superfamily of G protein-coupled receptors and is coupled to the stimulation of adenylyl cyclase. Upon continuous agonist stimulation, G protein-coupled receptors undergo a regulatory process termed homologous desensitization. Homologous desensitization requires a plethora of proteins such as specific G protein-coupled receptor kinases and arrestins. β-adrenergic receptor kinases (βARK) phosphorylate agonist-occupied receptors while arrestin proteins bind to phosphorylated receptors to uncouple the G protein from the receptor. This study was aimed at investigating the role played by βARKs and arrestins in homologous desensitization of the human D1A dopamine receptor expressed in human embryonic kidney cells (293). These cells were transfected transiently with the human D1A receptor alone or with the β-adrenergic receptor kinase 1 (βARK1) or β-adrenergic receptor kinase 2 (βARK2). Cells were pre-labeled with ^{32}P-inorganic phosphate and stimulated with 10 μM dopamine for 15 minutes. Phosphorylated receptors were immunoprecipitated and the extent of phosphorylation was determined by SDS-PAGE. Cells expressing the human D1A receptor alone displayed little agonist-dependent phosphorylation while cells overexpressing βARK1 or βARK2 showed an increase of 2 to 3 fold in agonist-dependent phosphorylation . These results suggest that the human D1A receptor may serve as a substrate for βARK1 and βARK2. We then investigated the role of βarrestin1 and βarrestin2 played in homologous desensitization of the human D1A receptor. In cells expressing D1A receptors alone, no desensitization to 10 μM dopamine (10 min.) was observed. In cells expressing βARK1 and βarrestin1, the desensitization of D1A receptors was slightly augmented and resulted in a 2 fold rightward shift in the dose-response curve for adenylyl cyclase activation with no decrease in the maximal activation. However, overexpression of βARK1 and βarrestin2 enhanced desensitization of the D1A receptor with a 3 fold rightward shift in dose-response curve and a reduction of the maximal activation of adenylyl cyclase (~20%). These data suggest that βarrestin2 confers a higher degree of desensitization of the human D1A receptor when coexpressed with βARK1. More studies will be performed using different receptor kinases and arrestin proteins to determine a speciciffity for these proteins in homologous desensitization of the D1A receptor.

I 171 A NOVEL, NEURONAL G PROTEIN-COUPLED RECEPTOR WITH HOMOLOGY TO THE LDL-RECEPTOR, H. van Heerikhuizen[1], C.P. Tensen[2], E.R. van Kesteren[1], R.J. Planta[1] and E.Vreugdenhil[1], Graduate School of Neurosciences Amsterdam, Research Institute Neurosciences Vrije Universiteit, [1]Faculty of Chemistry, Department of Biochemistry and Molecular Biology, [2]Faculty of Biology, Department of Organismic Zoology, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands.

Searching for G protein-coupled receptors (GPRs), we have isolated and characterized a cDNA from the central nervous system (CNS) of the mollusc, *Lymnaea stagnalis*, encoding a protein of 1115 amino acids with close sequence similarity to two different types of receptor proteins. Following a 20 residue long signal peptide, the N-terminal part of the protein contains two types of internal repeated sequences. The first type of repeat displays a high sequence similarity with the binding domains of the low density lipoprotein (LDL)-receptor, a characteristic thusfar only found in the LDL receptor-related protein (LRP) and in glycoprotein GP330. The second repeat, and the C-terminal part of this receptor are homologous to specific regions of a set of GPRs, the mammalian glycoprotein hormone receptors. The mRNA encoding this receptor (designated GRL101) is predominantly located in a cluster of 15-20 neurons within the CNS and to a lesser extent in the heart. The structural resemblance of the N-terminal part of GRL101 with the binding domains of the LDL receptor and its specific expression in a small number of neurons, suggest a role for lipoprotein-like molecules in neuronal, G protein-mediated signal transduction. Alternatively, GRL101 might be a receptor for ligands similar to those for LRP. This receptor was originally proposed to play a role in lipoprotein metabolism. Recently, however, it has become clear that LRP is a multiple ligand receptor, that can recognize and internalize additional, non-related ligands, *e.g.* uPA-PAI-1 complexes. Effects of uPA-PAI-1 and a related protein, tPA, on neuronal outgrowth and plasticity were recently reported and we therefore hypothesize that GRL101 might be the first member of a novel class of GPRs that directly transduces signals carried by large extracellular (lipo)protein(complexe)s into neuronal events mediated by G-proteins.

I 172 COMPUTATIONAL SIMULATION OF THE STRUCTURE, DYNAMICS, AND SIGNAL TRANSDUCTION MECHANISMS IN THE GPCR: THE 5-HT_2 AND 5-HT_{1A} RECEPTORS

Harel Weinstein, Daqun Zhang and Leonardo Pardo, Department of Physiology and Biophysics, Mount Sinai School of Medicine, New York, NY 10029

Molecular models were constructed for the transmembrane portions of the 5-HT_{1A} and 5-HT_2 subtypes of serotonin receptors--members of the GPCR family -- and subjected to quantum chemical (QC) and molecular dynamics (MD) simulations of the their complexes with ligands including full agonists, partial agonists, and antagonists (e.g., see Zhang & Weinstein, J. Med. Chem, 36:934-938; ibid. Med. Chem. Res., 1993 - in press). The strategy used for modeling incorporated previously proposed recognition sites (Weinstein & Osman, Neuropsychopharmacol., 3:397, 1990), and structure- activity relationships of ligand interactions with the receptor as the first criteria for model construction; the topological template for the helix bundle was determined primarily based on molecular biology experiments with chimeric GPCR, rather than on the template of BR that was shown to be inappropriate (see Pardo et al., PNAS 1992, 89:4009-4012) even before the observed differences from the rhodopsin topology. Results from quantum mechanical calculations of the complexes with ligands in the recognition sites of the receptors, as well as from MD simulations of ligand/receptor complexes reveal different patterns of ligand selectivity, and structural changes induced in the TMHs by the binding in the recognition sites of ligands with different pharmacological efficacies. These results lead to the identification of selectivity determinants, of molecular mechanisms that trigger receptor activation, and of a putative signal transduction mechanism. All these structural and functional characteristics were found to be consistent with the results of experimental probing of structure-function relationships in GPCR, and with the pharmacological efficacies of the ligands. The receptor model and the related mechanism of activation revealed from the computational simulations constitute novel insight at the atomic level of detail that can guide the detailed exploration of structure-function relations in GPCR, of the molecular details of mechanisms of signal transduction, and the design of new ligands with predetermined pharmacological properties. Supported by NIH grants DA-06620 and DA-00060.

I 173 GLIMPSES OF A G PROTEIN EFFECTOR'S STRUCTURE: TRANSMISSION ELECTRON MICROSCOPY OF RETINAL cGMP PHOSPHODIESTERASE, Theodore G. Wensel, Jacqueline Sines, and Justine A. Malinski, Verna & Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030

Little is known about the three dimensional structures of G protein-coupled effectors, and how these might interact with G proteins and with the membranes on which they carry out their functions in signal transduction. Those effectors which may not be readily accessible to x-ray crystallography due to lipophilic properties or difficulties in obtaining sufficient quantities can be viewed directly by electron microscopy. Recent advances in image processing technology allow useful information on protein structure to be extracted from non-crystalline samples as well as from monomolecular planar arrays. Analyses of electron images of purified cGMP phosphodiesterase have revealed its overall shape and size in projection. The appearance of multiple views of the protein on each grid allows low-resolution three dimensional models to be formulated and tested. Supported by NIH Grants EY07981 and RR02250.

I 174 DELETION ANALYSIS OF THE INSULIN RECEPTOR α-SUBUNIT: IMPLICATIONS FOR STRUCTURE AND FUNCTION

Jonathan Whittaker and Dennis Mynarcik. Department of Medicine, SUNY at Stony Brook, Stony Brook, NY 11794

Insulin binding to the a subunit of its receptor initiates conformational changes that lead to signal transduction. The structural basis for these changes is currently unknown. Recent evidence suggests that the insulin binding site(s) resides within the amino terminal 500 amino acids of the receptor.

Since it has proven possible to make extensive deletions between amino acids 470-590 without compromising the stability of the protein, we have undertaken a systematic deletion analysis of this region of the receptor to elucidate the structural basis of signal transduction. Deletion mutants were expressed in COS cells and the structure and function, including insulin binding and autophosphorylation, of the mutated receptors were evaluated. Such analyses have enabled us to identify an independently folded domain containing the insulin binding site and also to define the minimum structural requirements for activation of the receptor tyrosine kinase. The implications for the structural organization of the receptor molecule will be discussed

I 175 INDEPENDENT ACTIVATION OF NEUTRAL AND ACIDIC SPHINGOMYELINASES BY DISTINCT DOMAINS OF THE 55-kDa RECEPTOR FOR TUMOR NECROSIS FACTOR, Katja Wiegmann, Thomas Machleidt, Stefan Schütze, and Martin Krönke[1], Institut für Medizinische Mikrobiologie und Hygiene, Technische Universität München, Trogerstrasse 9, 81675 München, Germany

Two different types of sphingomyelinases (SMase), neutral N-SMase associated with the cell membrane and acidic A-SMase localized in endosomal and lysosomal compartments have been recognized as important enzymes in growth factor receptor signaling. We here show that both N-SMase and A-SMase can be activated simultaneously by one single cell surface receptor, the human 55 kDa receptor for tumor necrosis factor (TNF-R55). In U937 cells, TNF rapidly induced both N-SMase and A-SMase activities with slightly different kinetics. Expression of human TNF-R55 deletion mutants in a mouse pre-B cell line, 70Z/3, revealed that N-SMase and A-SMase are activated by distinct intracytoplasmic domains of TNF-R55, respectively, indicating that neither of these SMases is dependent on the other. While the acidic SMase can be shown to couple to the activation of the transcription factor NF-*κ*B, the neutral SMase segregates from the NF-*κ*B activation pathway yet coincides with inducibility of phospholipase A_2 activity. Although TNF-induced activation of either SMase generates the same second messenger-like molecule, ceramide, these data indicate that N-SMase and A-SMase functions are dissociable and depend on their respective subcellular location.

I 176 SCANNING MUTAGENESIS OF THE INSULIN RECEPTOR α-SUBUNIT LIGAND BINDING SITE

Paul Williams, Dennis Mynarcik and Jonathan Whittaker. Department of Medicine, SUNY at Stony Brook, Stony Brook, NY 11794

Exons 1,2, 4 and part of exon 5 of the insulin receptor gene are thought to encode the regions of the α-subunit of the receptor protein responsible for ligand binding. The construction of receptor chimeras and affinity labeling studies have implicated the first 68 amino acids of the n-terminus of the receptor in this function. In addition mutations in the c-terminal half of the α-subunit (aas 300-500) produce changes in affinity for insulin.

Charged to alanine scanning mutagenesis has been used to specifically map the amino acid residues involved in insulin binding. Charged and aromatic amino acids (in groups of 2-5) in the first 128 amino acids of the n-terminus of the receptor have been progressively mutated to alanine by site directed mutagenesis of the receptor cDNA and the mutant cDNAs were transiently expressed in COS-1 cells. The expressed mutant receptors were evaluated for their structure and function. The results of these studies will be discussed.

I 177 MODULATION OF THE FUNCTION OF MAMMALIAN RHODOPSIN BY MEMBRANE CHOLESTEROL

Philip L. Yeagle and Arlene D. Albert, Department of Biochemistry, University at Buffalo (SUNY), Buffalo, NY 14214

Rhodopsin function has recently been observed to be modulated by the cholesterol content of the membranes in which the rhodopsin resides. The membrane cholesterol content in the photoreceptor cell varies considerably among the membranes of the retinal rod cell. In the plasma membrane and in the newly formed disks, the cholesterol content is relatively high, while in the mature disks the cholesterol content is low [1]. The ability of rhodopsin to activate the cGMP cascade is much reduced in the membranes with high cholesterol content [2]. How membrane cholesterol modulates rhodopsin function has been investigated. Two hypotheses have been investigated. A fluorescent derivative of cholesterol, cholestatrienol, has been used to test the hypothesis of a direct sterol-rhodopsin interaction for modulation of rhodopsin function. While energy transfer experiments indicate some interaction between sterol and protein, the data do not support a direct, specific, sterol-protein interaction. Therefore another hypothesis for cholesterol modulation of rhodopsin function has been investigated, in which the cholesterol modifies the bulk properties of the membrane, which in turn, modulate the function of rhodopsin. Work from Litman's laboratory has implicated internal membrane free volume in rhodopsin function [3]. This internal volume can be shown to be modulated by cholesterol. This mechanism is dependent upon the organization of cholesterol in the membrane. We have therefore investigated the organization of cholesterol in the disk membrane by ^{13}C NMR of ^{13}C-labeled cholesterol. These data are supportive of the hypothesis of a cholesterol alteration of the lipid bilayer in which the receptor resides as a mechanism for modulation of rhodopsin function. (National Institutes of Health EY03328)

1. Boesze-Battaglia, Hennessey & Albert 1989 J. Biol. Chem. 264, 815.
2. Boesze-Battaglia & Albert. 1990 J. Biol. Chem. 265, 20727
3. Mitchell, Straume, Miller & Litman 1990 Biochemistry 29, 9143.

I 178 THROMBIN RECEPTOR/EFFECTOR COUPLING IS MEDIATED BY DISTINCT G_i PROTEINS IN CHO CELLS,

S. Zaina*, S. Law* and J. Stadel*#, *Dept. of Pharmacology, Univ. of Pennsylvania., Philadelphia. PA 19104 and #SmithKline Beecham, King of Prussia, PA 19406.

Cellular signaling by thrombin is initiated by proteolytic activation of surface receptors and tranduced across the plasma membrane by G proteins. Cellular responses to thrombin involve the modulation of a variety of effectors and responses are affected by pertussis toxin (PTX) to variable degrees suggesting that G_i and/or G_o contribute to thrombin responses. To investigate the specificity of G protein mediated signal transduction, we used a subclone of chinese hamster ovary (CHO) cells that expresses thrombin receptors and detectable levels of only $G_{o\alpha}$ and $G_{i\alpha3}$ among PTX substrates. Thrombin mobilized Ca^{2+} in a PTX-sensitive manner in these cells, while none of the other responses tested (PLA_2 stimulation, adenylyl cyclase inhibition or protein synthesis) could be detected. To determine whether the presence of $G_{i\alpha1}$ or $G_{i\alpha2}$ is essential to gain the different cellular responses to thrombin, CHO cell lines stably expressing either $G_{i\alpha1}$ or $G_{i\alpha2}$ were obtained. Thrombin stimulated PLA_2 activity only in CHO cells transfected to express $G_{i\alpha2}$; the response was completely blocked by PTX and by the specific thrombin inhibitor, hirudin. In membranes prepared from cells expressing $G_{i\alpha1}$, but not $G_{i\alpha2}$, thrombin inhibited forskolin-stimulated adenylyl cyclase (28.7±1.3%). PTX and hirudin both suppressed the response. When challenged for their ability to stimulate protein metabolism in response to thrombin, as measured by [^{3}H]leucine incorporation, both the CHO cell lines expressing $G_{i\alpha1}$ or $G_{i\alpha2}$ showed a positive response compared to vector transfected control cells. Thrombin alone produced little or no response, but a synergy with low doses of basic fibroblastic growth factor (bFGF) was observed. In the presence of PTX, the response was lowered to the level of bFGF alone. These data show that the pleiotropic cellular responses evoked by thrombin are mediated by the activation of a complex network of G proteins that direct signals to distinct effectors in CHO cells.

Poster Session II

I 200 v-FPS CONSTITUTIVELY ASSOCIATES WITH THE PDGF RECEPTOR AND INDUCES RECEPTOR PROTEIN DOWN REGULATION

Deborah H. Anderson, Cancer Research Unit, Saskatoon Cancer Centre, 20 Campus Drive, Saskatoon, Saskatchewan, Canada S7N 4H4

c-Fps/c-Fes is a cytoplasmic tyrosine kinase which contains an SH2 domain, but no SH3 domain, in addition to the catalytic sequences. It's expression is restricted to hematopoietic cells. The transforming v-Fps protein has a virally derived gag sequence fused to the amino-terminus of the protein and it's tyrosine kinase activity is constitutively activated. Mutations or deletions in the SH2 domain of v-Fps have been shown to impair the transforming ability of the protein in Rat-2 fibroblast cells. The mechanism by which *v-fps* transforms Rat-2 fibroblasts is not known.

Immunoprecipitates of the endogenously expressed PDGF receptor protein from fibroblasts transformed by *v-fps*, also contain the v-Fps protein. This association between v-Fps and the PDGF receptor is independent of the presence of the v-Fps SH2 domain, since an SH2 deletion mutant will also coimmunoprecipitate with the receptor. Additionally, this association is independent of prior growth factor stimulation with PDGF, since it is also observed in fibroblasts prior to PDGF stimulation. The portion of the v-Fps protein required for mediating it's association with the PDGF receptor, as indicated above, is not the Fps SH2 domain, instead the kinase domain and/or kinase activity appears to be the important feature.

PDGF mediated mitogenesis is severely reduced in *v-fps* transformed cells, in large part because the level of PDGF receptor protein is extremely low. This down regulation of receptor levels is likely due to constitutive activation of the PDGF receptor, followed by receptor internalization and degradation. Attempts to reconstitute the association between the PDGF receptor and the v-Fps protein have demonstrated the requirement for at least one other cellular component. Thus, this association appears to be indirect, via at least one other unidentified protein intermediate.

We will also discuss a model for *v-fps* transformation, whereby association of v-Fps either allows it to phosphorylate the PDGF receptor directly, or activate the endogenous receptor kinase activity. This in turn activates PDGF receptor signal transduction pathways, resulting in continuous mitogenesis and cellular transformation, as well as PDGF receptor down regulation.

I 201 PHOSPHOTYROSINE DEPENDENT IMMUNOPRECIPITATION OF PTDINS 3-KINASE (PI3K) FROM ACTIVATED PLATELETS IN THE ABSENCE OF PI3K TYROSINE PHOSPHORYLATION. ASSOCIATION OF PI3K WITH A PHOSPHOTYROSYL PROTEIN. James A. Augustine and Norman R. Geltz. The Laboratory of Cell Biology, Immunogenetics Research Section, The Blood Center of Southeastern Wisconsin, Milwaukee, WI, 53233.

Agonist-induced intracellular signaling events are crucial to the process of platelet activation. Protein tyrosine phosphorylation and phosphatidylinositol 3-kinase (PI3K) activation are two very rapid signaling events induced in thrombin-activated platelets. In this study, we explore thrombin receptor-directed signaling of intracellular protein tyrosine phosphorylation and its potential role in PI3K activation in human platelets using the thrombin receptor "tethered ligand" synthetic peptide, SFLLRNPNDKY, a platelet activation agonist that mimics the activated thrombin receptor. We show that SFLLRNPNDKY induces a concentration-dependent pattern of intracellular protein tyrosine phosphorylation identical to that of thrombin. Anti-phosphotyrosine (anti-Ptyr) antibody immune complexes from detergent-soluble lysates of SFLLRNPNDKY-activated platelets contained increased levels of p85, the regulatory subunit of PI3K, and PI3K enzymatic activity as determined by anti-p85 immunoblotting and PI3K enzyme assays. Paradoxically, anti-Ptyr immunoblot analysis of anti-p85 monoclonal antibody immune complexes showed that the p85 or p110 subunits of platelet PI3K were not phosphorylated on tyrosine either in the absence or presence of platelet activation with SFLLRNPNDKY. The anti-Ptyr immunoblot did, however, reveal the presence of stimulus-dependent increases in the levels of 63 and 68 kDa phosphotyrosyl proteins co-immunoprecipitating with p85. These results indicate that thrombin receptor-directed transmembrane signaling of PI3K activation in platelets does not involve direct tyrosine phosphorylation of PI3K, but rather, involves tyrosine phosphorylation of a protein that is either associated with or becomes associated with PI3K.

I 202 ZYXIN: AN ADHESION PLAQUE PROTEIN THAT EXHIBITS A ZINC FINGER MOTIF FOUND IN CERTAIN TRANSCRIPTION FACTORS. Mary C. Beckerle, Aaron Crawford, James Michelsen, Ingrid Sadler, Karen Schmeichel, and Dennis Winge*. Departments of Biology and Biochemistry*, University of Utah, Salt Lake City, UT 84112

Interaction with extracellular matrix can trigger a variety of responses by cells including cell differentiation. The mechanism by which cell surface events are coupled to the transcriptional machinery is not understood, however certain proteins localized at sites of cell-substratum contact are likely to function as signal transducers. We have recently identified and characterized a low abundance adhesion plaque protein called zyxin. Zyxin exhibits an unusual proline-rich N-terminal region followed by three tandemly arrayed LIM domains. The LIM domain , which exhibits the consensus sequence $CX_2CX_{16-23}HX_2CX_2CX_2CX_{16-21}CX_{2-3}$(C,H,D), was first identified in the homeodomain proteins Lin-11, Isl-1, and Mec-3, each of which plays a role in control of gene expression and cell differentiation. To learn more about the function of zyxin, we have carried out a biochemical screen to identify zyxin binding partners. Interestingly, we have discovered that zyxin interacts *in vitro* with the Cysteine-Rich Protein (CRP), an evolutionarily conserved 23 kDa protein that also exhibits LIM domains. Zyxin and CRP are extensively co-localized *in vivo*, consistent with the conclusion that the association we detect *in vitro* has physiological relevance. We have begun to characterize the structure and function of the LIM domain. We have demonstrated that the LIM domain is a specific zinc-binding domain that coordinates two metal ions. Metal binding is required to establish the tertiary fold of the LIM domain. To examine whether the LIM domain functions as a protein binding interface, we have performed domain mapping studies to define the region of zyxin that interacts with CRP. We have found that the LIM region of zyxin is both necessary and sufficient to support zyxin's association with CRP. Our results support the hypothesis that LIM domains can function in protein-protein interaction. The demonstration that two cytoskeletal proteins, zyxin and CRP, share a sequence motif with proteins important for transcriptional regulation raises the possibility that zyxin and CRP are components of a signal transduction pathway that mediates adhesion-stimulated changes in gene expression.

I 203 A VIRAL PROTEIN IS ASSOCIATED WITH $p56^{lck}$ IN HUMAN T cellS TRANSFORMED BY HERPESVIRUS SAIMIRI. Brigitte Biesinger[1], Alexander Y. Tsygankov[2], Helmut Fickenscher[1], Frank Emmrich[3], Bernhard Fleckenstein[1], Joseph B. Bolen[2], and Barbara M. Bröker[3,4]. [1]Institut für Klinische und Molekulare Virologie, Universität Erlangen-Nürnberg, Loschgestr. 7, D-91054 Erlangen, [2]Bristol-Myers Squibb, Department of Molecular Biology, Signal Transduction Laboratory, Princeton, NJ 08543, [3]Max-Planck-Gesellschaft, Schwabachanlage 10, D-91054 Erlangen, [4]Bernhard-Nocht-Institut für Tropenmedizin, Bernhard-Nocht-Str.74, D-20359 Hamburg

Human T cells transformed to permanent growth by Herpesvirus saimiri are a new model system for activated T cells. They express the alpha/beta T cell receptor and either CD4 or CD8 at their surface and produce cytokines as expected for cells of their phenotype. Their response to stimulation via CD3 and/or CD4 does not differ from that of untransformed activated T cells with regard to protein tyrosine phosphorylation and calcium mobilization. Specific antigen recognition is preserved when antigen-specific T cell clones are transformed. Studying early signalling events in these transformed cells we found a novel 40kD phosphoprotein associated with the T cell specific tyrosine kinase $p56^{lck}$. With the help of an anti-peptide antiserum, in vitro translation, and a bacterial fusion protein we could identify pp40 as a viral gene product. This protein binds to human $p56^{lck}$ and is phosphorylated by the enzyme. Therefore it possibly plays an important role in the T cell specific transformation by Herpesvirus saimiri. Its function might also provide some new insights into T cell activation.

I 204 BIOSYNTHESIS AND SIGNALLING FUNCTION OF (PRE-) B CELL RECEPTOR COMPLEXES, J. Borst, G. Brouns and E. de Vries, Div. of Cellular Biochemistry, The Netherlands Cancer Institute, Amsterdam, The Netherlands

The B cell receptor (BCR) complex consists of immunoglobulin (Ig) heavy and light chains and a disulfide linked heterodimer of Igα and Igß. These latter two proteins are encoded by the B cell specific genes mb-1 and B29 and contain tyrosine motifs, that after phosphorylation may bind to SH2 domain containing enzymes, such as *src*-related tyrosine kinases, which transduce the signal initiated by receptor triggering. Expression of Ig genes plays a crucial role in B cell development. In pre-B cells, the transmembrane form of μ heavy chain is required for B cell survival and regulation of allelic exclusion. In mature B cells, Ig molecules are essential for induction of proliferation and differentiation. We are addressing the question what the molecular basis for this differential signalling is.

To this end, we compared the structure of receptor complexes expressed on pre-B and mature B cells. Like the mature BCR, the pre-BCR is associated with the Igα/ß heterodimer. Both pre-BCR and mature BCR are associated with tyrosine- and serine/threonine kinases. Association of μ heavy chain with the Igα/ß heterodimer occurs in the ER, as read out by the acquisition of Endo H resistance. In mature B cells, transport out of the ER occurs efficiently once the complex is complete. In pre-B cells, exit of assembled complexes from the ER is at least tenfold less efficient, although μ chain, Igα and Igß associate rapidly. To investigate at what timepoint during *biosynthesis kinases* associate with the BCR, we separated ER and plasma membrane localized molecules by lectin purification. From both pools of glycoproteins, (pre-)BCR complexes were isolated and subjected to *in vitro* kinase assays. In pre-B cells, the majority of kinase activity is associated with the large immature protein pool, whereas in mature B cells the complex glycosylated fraction contains most kinase activity.

ER retention of pre-BCR complexes may be mediated by the heavy chain binding protein BiP, that may be more efficiently displaced by conventional light chain than by pseudo light chain complexes. However, involvement of another, pre B cell specific, retention mechanism cannot be excluded. No ligand for the pre-BCR has been identified and in view of the data reported here a mode of action of this receptor complex could be intracellular signalling from the ER as a consequence of receptor aggregation.

I 205 VAV INTERACTS WITH A HIGH MOLECULAR WEIGHT GTP-BINDING PROTEIN BUT IS NOT A GDP-RELEASING FACTOR FOR RAS PROTEINS. Xosé R. Bustelo, Ki-Ling Suen and Mariano Barbacid, Department of Molecular Biology, Bristol-Myers Squibb Pharm. Research Inst., Princeton, NJ, 08543-4000.

The product of the *vav* proto-oncogene, Vav, is a SH2/SH3-containing protein which exhibits a series of structural motifs including a leucine-rich domain, a cysteine rich region and a Dbl-homology (DH) domain. Previous studies have shown that Vav ectopically expressed in NIH3T3 cells becomes associated with and is phosphorylated by ligand-activated PDGF and EGF receptors. More importantly, engagement of the T-cell TCR/CD4 complex and the mast-cell receptor leads to the rapid phosphorylation of the endogenous Vav on tyrosine residues. Similar results have been obtained in c-Kit-expressing cells upon addition of *Steel* factor and in B-cells following activation of their IgM antigen receptors. Although the involvement of Vav in hematopoietic signal transduction has been well illustrated, its precise function remains obscure. It has been recently reported that Vav is the major guanosine nucleotide releasing factor (GRF) for Ras in hematopoietic cells [Gulbins *et al.*, Science 260, 822 (1993)]. The following observations are inconsistent with such a role: i) Vav proteins display no GRF activity toward Ras in standard *in vitro* assays; ii) cells overexpressing Vav do not have increased levels of GTP-bound Ras proteins, iii) transformation of NIH3T3 cells by the *vav* oncogene is not inhibited by a farnesyl transferase inhibitor that completely blocks *ras* transformation; iv) Vav does not overcome the growth inhibitory activity of RasN17, a dominant negative mutant that blocks Ras signaling by inhibiting Ras GRFs. In a search for Vav associated proteins we have identified a 68 kDa GTP-binding protein that is constitutively associated with Vav. This association is mediated by the DH and SH3 domains, but not by the SH2 region of Vav. These observations suggest that Vav may be part of a signaling pathway that involves a new class of high molecular weigh GTP-binding proteins.

I 206 MOLECULAR CLONING AND SEQUENCING OF A 58 KDA MEMBRANE-AND MICROFILAMENT-ASSOCIATED *gag*-LIKE PROTEIN FROM ASCITES TUMOR CELL MICROVILLI WHICH BINDS SRC SH3 DOMAIN. C.A. Carothers Carraway,[1] S.-H. Juang,[1] Y. Li,[1] J. Huang,[1] A. Gallo,[1] B.J. Mayer,[3] N. Fregien,[2] and K. L. Carraway.[2] Depts. of Biochemistry & Molecular Biology[1] and Cell Biology & Anatomy,[2] Univ. of Miami School of Medicine, Miami, FL 33136 and The Rockefeller Institute,[3] New York, NY 10021

Ascites sublines of the 13762 rat mammary adenocarcinoma provide a useful model system for investigating membrane-microfilament (MF) interactions. The MAT-C1 subline differs from the MAT-B1 subline in having highly stable, branched microvilli (MV) and immobile cell surface receptors. The sublines differ by a 58 kDa protein (58K), present in MAT-C1 MV in a transmembrane complex (TMC) with actin and a high M_r complex of at least five glycoproteins (55, 65, 80, 110 and 120 kDa) [C. Carraway et al. (1983) Proc. Natl. Acad. Sci. 80, 430-434); C. Carraway et al. (1991) J. Biol. Chem. 266, 16238-16246]. The TMC serves as a core for a large signal transduction particle containing $p185^{neu}$ [C. Carraway et al. (1993) J. Biol. Chem. 268, 5582-5587] and several known signal transduction components, including Src. Purified 58K binds phospholipids and blocks actin polymerization, acting as a filament capping protein. These membrane- and MF-binding properties are consistent with the proposed role of 58K in stabilizing the highly malignant MAT-C1 cell surface by stabilizing the interactions between the microvillar MFs and the membrane. A MAT-C1 cDNA library was screened with a degenerate oligonucleotide derived from the sequence of a tryptic peptide of 58K, and overlapping cDNAs of 0.6 and 0.8 kb were isolated from the 3' end of the 58K transcript. Additional cDNAs were cloned using primers from the 5' end of the 0.8 kb cDNA and the RACE protocol for PCR amplification of cDNA ends. The complete sequence derived from these combined cDNAs showed a surprising similarity to mammalian retroviral *gag* proteins, including regions corresponding to p15, p12 and the N-terminal 80% of p30. 58K and the corresponding regions of the *gag* proteins for Moloney murine leukemia virus indicated about 50% identity of their amino acid sequences. 58K also contained a sequence (PPPYPVPTAPP) within a longer proline-rich sequence similar to those found in Src and Abl SH3 domain-binding proteins 3BP1 and 3BP2. 58K bound to Src SH3 domain-agarose, and *in vitro*-translated 58K co-immunoprecipitated with platelet c-Src. These results suggest that 58K is an important element in organizing a MF-associated signal transduction particle at the cytoplasmic surface of the plasma membrane in the ascites cells. We propose that binding of 58K to Src may also modulate Src activity and transduction of signal in the tumor cells.

I 207 SYNERGISTIC RESPONSE OF $p185^{erbB2/neu}$ AND $p180^{erbB3}$ TO HEREGULIN-ß1, Kermit L. Carraway III†, Robert W. Akita#, Lewis C. Cantley†, Richard A. Cerione*, and Mark X. Sliwkowski#; †Harvard Medical School and Division of Signal Transduction, Beth Israel Hospital, Boston, MA 02115; #Department of Protein Chemistry, Genentech, Inc., South San Francisco, CA 94080; *Department of Pharmacology, Cornell University, Ithaca, New York 14853.

The heregulins (HRGs) were originally identified as candidate ligands for $p185^{erbB2/neu}$ on the basis of their ability to stimulate the tyrosine phosphorylation of this receptor in human cancer cells. Recent data indicate that heregulin does not bind to $p185^{erbB2/neu}$ in fibroblasts transfected with the *erbB-2* cDNA, suggesting that another receptor present in cancer cells is responsible for HRG-induced effects. We have found that [^{125}I]HRGß1 binds to insect cell-expressed bovine $p180^{erbB3}$ with a K_d of ~1 nM, indicating that $p180^{erbB3}$ is a primary receptor for HRGß1. We have observed that a monoclonal antibody specific for $p185^{erbB2}$ lowers the affinity of some cancer cell receptors for [^{125}I]HRGß1 by 3-7 fold, and blocks HRGß1-stimulated tyrosine phosphorylation of receptors in the 180-190 kDa region. These data suggest that $p180^{erbB3}$ and $p185^{erbB2}$ respond synergistically to HRGß1. We have also observed that COS cells co-transfected with both $p185^{erbB2}$ and $p180^{erbB3}$ bind [^{125}I]HRGß1 ~10 fold more tightly than cells transfected with $p180^{erbB3}$ alone. Together, these data are consistent with the hypothesis that a $p180^{erbB3}/p185^{erbB2}$ heterodimer forms a high affinity binding site for HRGß1, and that the formation of a heterodimeric receptor species is necessary for transducing a signal across the membrane.

I 208 THE α AND β SUBUNITS OF THE TYPE I IFN RECEPTOR ASSOCIATED WITH THE p135^{tyk2} TYROSINE KINASE AND WITH THE TRANSCRIPTIONAL ACTIVATOR ISGF3α, O. R. Colamonici, J. Krolewski, X-Y. Fu, and P. Domanski. Dept. of Pathology, Univ. of Tennessee, CPMC, and Mount Sinai Hospital.

The Type I IFN receptor (IFN-R) has a multichain structure formed by subunits with MW of 110 (α subunit), 100 (β subunit) and 75 (cloned receptor subunit) kDa. After Type I IFN binding to the receptor, three of the proteins that form the transcriptional activator ISGF3 are activated by tyrosine phosphorylation. The p135^{tyk2} kinase has been found to be part of the Type I IFN signal transduction pathway. We have demonstrated that p135^{tyk2} associates with the α subunit of the Type I IFN-R. Tyrosine phosphorylation of the α subunit of the receptor and p135^{tyk2} have similar time courses (Colamonici and Krolewski, J.B.C. in press). We have also studied the protein-protein interactions between the α and ß subunits of the receptor, p135^{tyk2}, and the different components of ISGF3α. Affinity crosslinking of 125I-IFN to the receptor followed by immunoprecipitation with specific anti-p113 and anti-p135^{tyk2} show that both p135^{tyk2} and the 113 component of ISGF3α are associated with the α and β subunits of the Type I IFN-R. These associations have also been observed after Western blot analysis indicating that the interaction between the receptor subunits and the 113 component of ISGF3α is detected before and after IFNα treatment. The association between p135^{tyk2} and the 113 component of ISGF3α, and the role of ISGF3 in the IFNα signal transduction are currently being explored.

I 209 DIFFERENTIATION AND THE CELL CYCLE: CDC37 FUNCTIONS IN SIGNALLING BY THE SEVENLESS TYROSINE KINASE RECEPTOR, Tyler Cutforth and Gerald M. Rubin, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

The R7 photoreceptor cell requires the activity of Sevenless, a receptor tyrosine kinase, for adoption of the neuronal fate. Mutations which dominantly enhance the phenotype of a barely functional Sevenless protein have been identified in seven genes, which are likely to define components of the signal cascade employed by Sevenless. The products of three of these genes (*Sos*, *drk* and *Ras1*) provide a direct link between Sevenless and Ras proteins, and are common signalling molecules for three tyrosine kinase receptors (*sev*, *Egfr* and *torso*) in *Drosophila*.

In contrast, mutations in *cdc37* act specifically in Sevenless signalling. This gene codes for a hydrophilic protein 23% identical over most of its length to the Cdc37 protein from *S. cerivisiae*, which was identified in a screen for mutations which arrest the cell cycle at START. The *Drosophila* protein rescues *cdc37* mutant yeast, indicating that its function is conserved between species. In addition, *cdc37* exhibits specific genetic interactions with mutations in the gene encoding p34^{cdc2}, the central protein kinase regulating cell cycle progression, in both yeast and *Drosophila*.

The R7 precursor cell is derived from a pool of undifferentiated epithelial cells which have recently completed a final mitotic division. We propose that terminal differentiation of the R7 cell requires exit from G_1 into a quiescent state, and that Cdc37 is involved in this decision to exit the cell cycle. The specificity of Cdc37 for Sevenless may reflect distinct responses to differentiative and proliferative signals during development.

I 210 ACTIVATION AND TRANSLOCATION OF MEMBRANE-ASSOCIATED PROTEIN KINASES BY PDGF.

Julie deBlaquiere, Francesca Walker, Valdo P. Michelangeli#, Louis Fabri and Antony W. Burgess. Ludwig Institute for Cancer Research, Melbourne, Australia, 3050 and #Department of Diabetes and Endocrinology, Royal Melbourne Hospital, Australia, 3050.

Binding of the growth factor PDGF to its receptor stimulates numerous intracellular processes which begin with the activation of the receptor tyrosine kinase and culminate in mitogenesis. Whilst some of the cytosolic protein kinases associated with the PDGF signalling cascade have been identified, the initial membrane events remain unclear. We have developed a system utilising isolated plasma membranes from NRK and Balb/c 3T3 cells which allows us to investigate the early effects of growth factor stimulation. Activation of the PDGF receptor tyrosine kinase causes the specific release of approximately 10 phosphoproteins from the plasma membrane. Most, but not all, of the observed phosphorylation is on serine/threonine residues and we have unequivocally identified the major kinase responsible as the cAMP-dependent protein kinase (PKA). Rp-cAMPs, a competitive cAMP analogue, abolishes cAMP-induced dissociation of PKA from the membrane, but does not inhibit the PDGF-mediated activation of PKA. Furthermore, although neither cell-line accumulates cAMP in response to PDGF, the level of PKA in the cytosol increases after stimulation with PDGF, suggesting a novel mechanism of activation for PKA by PDGF. We have also shown that at least one tyrosine kinase, pp60$^{c\text{-}src}$, is specifically activated in response to PDGF. We have now identified PKA as the kinase responsible for the amino-terminal phosphorylation and concomitant release of pp60$^{c\text{-}src}$ from the plasma membrane. Elucidation of the mechanism by which the PDGF receptor stimulates the release of PKA is currently under study in our laboratory.

References

deBlaquiere, J., Walker, F., Michelangeli, V.P., Fabri, L., and Burgess, A.W. PDGF Stimulates the Release of PKA from the Cell Membrane, 1993, *J. Biol. Chem.*, In Press.

Walker, F., deBlaquiere, J., and Burgess, A.W. Translocation of pp60$^{c\text{-}src}$ from the Plasma Membrane to the Cytosol after Stimulation by PDGF, 1993, *J. Biol. Chem.*, In Press.

I 211 PI3 KINASE MEDIATES bFGF- AND SERUM-STIMULATED SMOOTH MUSCLE CELL GROWTH, Larry Denner, Ajay Rege, Lydia Sturgis, Nikki Kint, Richard Dixon and Cliff Stephan, Departments of Pharmacology, Cell Biology and Chemistry, Texas Biotechnology Corporation, Houston, TX 77030

Smooth muscle cell (SMC) proliferation plays an important role in normal and pathophysiological responses in the vasculature. We have used phosphorothioate antisense oligonucleotides (AS-ODN) to study the role of the catalytic subunit (p110) of phosphatidylinositol 3'-kinase (PI-3K) in regulation of SMC growth. Primary rat carotid SMCs were growth arrested in low serum for 4 days. Subsequent treatment with 10% serum for 3 days stimulated growth about 4 fold. This growth was blocked by treatment with p110 AS-ODN, but was unaffected by AS-ODN to many other mRNAs such as PKC epsilon, lamininA, and vinculin. RT-PCR analysis indicated that p110 AS-ODN almost completely blocked serum-stimulated p110 mRNA expression while not affecting mRNA for vinculin or the housekeeping gene, GAPDH. Further, PI-3K activity in serum-stimulated SMCs was inhibited by p110 AS-ODN treatment (70%, p,0.01). bFGF, in the presence of serum, stimulated growth about 50% more than serum alone. The growth stimulatory effects of both the serum and bFGF components were blocked by p110 AS-ODN. PDGFa receptor AS-ODN inhibited growth stimulated by serum but not by bFGF. These results suggest a necessary and specific role for PI3 kinase in the mitogenic response of SMCs to serum and bFGF.

I 212 FUNCTIONAL STUDIES OF THE ACTIVATION OF THE PLATELET-DERIVED GROWTH FACTOR RECEPTOR BY THE BOVINE PAPILLOMA VIRUS E5 PROTEIN, Daniela Drummond-Barbosa and Daniel DiMaio, Department of Genetics, Yale University School of Medicine, New Haven, CT 06510

Our laboratory has shown that the E5 protein of the bovine papilloma virus (BPV-E5) is able to cause morphologic and tumorigenic transformation of mammalian cells in culture through binding to and activation of the platelet-derived growth factor (PDGF) receptor.

We have developed a functional assay to study activation of the PDGF receptor pathway by BPV-E5 by using the Ba/F3 lymphoid cell line. Parental Ba/F3 cells are strictly IL-3-dependent for survival and growth and do not normally express PDGF receptor. We have used recombinant retroviruses expressing BPV-E5 and human PDGF-ß receptor to introduce these genes into Ba/F3 cells (the PDGF receptor retroviruses were obtained from Dr. Andrius Kazlauskas). Stable co-expression of the wild type PDGF receptor and BPV-E5 in Ba/F3 cells allows them to grow in an IL-3-independent fashion. In contrast, expression of neither protein alone enables these cells to grow in the absence of IL-3. In addition, we have demonstrated that BPV-E5 expression in Ba/F3 cells causes increased tyrosine phosphorylation of the mature form of the PDGF receptor and also novel phosphorylation of the immature precursor form of the receptor. We have also detected a stable complex between BPV-E5 and both forms of the receptor in this system by the co-immunoprecipitation technique.

Using this functional assay, different PDGF receptor mutants can be tested for their ability to mediate BPV-E5 action. We have shown that co-expression of BPV-E5 and a tyrosine kinase negative PDGF receptor mutant (K635R) does not allow IL-3-independent growth of Ba/F3 cells, even though the kinase negative receptor is expressed at levels comparable to wild type. This result shows that the tyrosine kinase activity of the receptor is important for its ability to mediate BPV-E5 activity. Additional PDGF receptor mutants in the process of being studied include phosphorylation site mutants and an amino-terminal truncated PDGF receptor.

I 213 INTERACTION OF HUMAN P56^{lck} SH2 DOMAIN WITH THE ZAP-70 TYROSINE KINASE, Pascale Duplay, Margot Thome, Frédérique Hervé and Oreste Acuto, Institut Pasteur, Laboratory of Molecular Immunology, Department of Immunology, 25/28 rue du Dr Roux, 75015 PARIS, France.

There is increasing evidence that p56lck plays an important role in signal transduction during T cell activation. p56lck contains an SH_2 domain found in a number of proteins involved in intracellular signaling including the src family of tyrosine kinases. This domain has been implicated in mediating protein-protein interaction by binding a tyrosine phosphorylated sequence on target proteins.

In order to evaluate further the function of p56lck and, in particular, its potential substrates, we used a recombinant p56lck SH2 domain to analyze the pattern of SH2-binding proteins from lysates of Jurkat cells before and after activation In non-activated Jurkat, we detected two tyrosine phosphorylated proteins which bound specifically to p56lck SH2. Upon activation for 1-2 minutes with anti-CD3 or anti-CD2 mAbs, seven additional tyrosine phosphorylated SH2-binding proteins were found. We identified the ζ-associated tyrosine kinase ZAP-70 as one of the proteins capable of specifically binding to the p56lck SH2 domain after CD3 stimulation. The significance of this interaction was further investigated *in vivo*. In agreement with the *in vitro* findings, p56lck could be coprecipitated with the ζ/ZAP-70 complex and conversely, ZAP-70 was detected in p56lck immunoprecipitates in activated Jurkat cells.

In addition to ZAP-70 other phosphorylated proteins were able to bind to the p56lck SH2 domain upon T-cell activation. Studies are in progress to identify these p56lck SH2-binding proteins.

I 214 Transformation by the mSos1 Ras exchange protein Sean E. Egan+^, Elizabeth M. C. Fisher#, and Julian Downward^, +Division of Immunology and Cancer Research, The Hospital for Sick Children, 555 University Ave. Toronto, Ontario, Canada, M5G-1X8. # Department of Biochemistry and Molecular Genetics, St. Mary's Hospital Medical School, Norfolk Place, London W2 1PG, United Kingdom, Signal Transduction Laboratory, The Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX, United Kingdom.

pBabeHDSos, a retrovirus expressing both the Hygromycin resistance gene and the Drosophila Sos gene can transform Rat1 fibroblasts. In contrast, the mSos1 expressing retrovirus pBabeHmSos1 does not transform Rat1 cells. In order to study the growth regulatory properties of the mammalian Sos1 gene, we have generated an extremely high titer retrovirus termed pMFGmSos1 (based on pMFG; Dranoff et al., 1993, PNAS). pMFGmSos1 is capable of transforming Rat1 cells at a low but reproducible frequency. Cloned transformed cell lined were generated from the pMFGmSos1 derived foci. These lines expressed extremely high levels of the mSos1 protein. Transfection of pZAP (replication competent clone of MoMuLV) into these cell lines resulted in the production of viral stocks which were highly transforming. Southern blot analysis was performed on the original pMFGmSos1 virus-transformed cell lines, and these lines were found to contain single integrated copies of the mSos1 provirus with no evidence of viral rearrangement. Size selected genomic libraries are being constructed in order to clone and characterize the integrated mSos1 alleles. These experiments will provide evidence for either mSos1 transformation via overexpression or mSos1 transformation via retrovirally induced mutation.

I 215 A UNIQUE H7-SENSITIVE PROTEIN KINASE IS IMPLICATED IN THE SIGNAL TRANSDUCTION PATHWAY OF *BORDETELLA PERTUSSIS* TRACHEAL CYTOTOXIN, Tod A. Flak, Linda Nixon Heiss and William E. Goldman, Department of Molecular Microbiology, Washington University, St. Louis, MO 63110

Tracheal cytotoxin (TCT) is a 921-dalton glycopeptide produced by *Bordetella pertussis*, the etiologic agent of whooping cough. This molecule is capable of reproducing the specific ciliated cell destruction observed in *B. pertussis* infection of respiratory epithelium. In a cell culture model employing primary hamster trachea epithelial cells, TCT combined with bacterial lipopolysaccharide (LPS) inhibits DNA synthesis. Key events in the mechanism of toxicity include the induction of intracellular interleukin-1α (IL-1α) and the subsequent generation of nitric oxide. Nitric oxide is an essential mediator in the toxicity, since specific inhibitors of nitric oxide production abrogate the destruction of ciliated cells in tracheal tissue and prevent the inhibition of DNA synthesis in HTE cells.

To further delineate the mechanism of TCT toxicity, we wished to identify the signaling events leading to the transcriptional activation of IL-1α. We determined that the broad-specificity kinase inhibitor H7 can completely block IL-1α mRNA production and the subsequent toxic effects induced by the TCT/LPS combination in respiratory epithelial cells. In addition, phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C (PKC), can reproduce the effects of TCT. However, the PKC-specific inhibitor calphostin C, while capable of blocking the effect of PMA, is not able to block the action of TCT. Likewise, the activation of PKC, assessed by translocation of PKC activity to the membrane fraction, is readily apparent in PMA-treated cells, but is not observed in TCT-treated cells. Moreover, other protein kinase inhibitors having greater selectivity for calmodulin-, cAMP-, or cGMP-dependent kinases (W7, A3, HA1004) do not block TCT/LPS toxicity. We conclude that the early signal transduction events in the mechanism of TCT toxicity may involve a unique protein kinase.

I 216 ACTIVATION OF SRC-FAMILY PROTEIN TYROSINE KINASES AND PHOSPHATIDYLINOSITOL-3 KINASE IN 3T3-L1 MOUSE PREADIPOCYTES BY INTERLEUKIN-11, Douglas K. Fuhrer, Anne L. Burkhardt, Joseph B. Bolen, Yu-Chung Yang. Bristol-Myers Squibb Research Institute, Department of Molecular Biology, Princeton, NJ 08543, Walther Oncology Center, Indiana University School of Medicine Indianapolis, IN 46202.

Interleukin (IL)-11 is a multifunctional cytokine involved in cellular proliferation and differentiation. Tyrosine phosphorylation is a part of the IL-11 signal transduction and response mechanism. When IL-11 is added to responsive cells the signal transducer gp130 is tyrosine phosphorylated in addition to a number of unidentified proteins. In order to determine whether Src-family tyrosine kinases are involved in this system, an IL-11 responsive cell line (3T3-L1, mouse preadipocyte cells) was tested for the presence of Src-family protein tyrosine kinases. In this cell line Yes, Fyn and Src were found to be expressed. Upon stimulation with IL-11, Yes is activated within 10 seconds where it reaches its peak activation. Src is activated and peaks at 30 seconds after IL-11 addition and Fyn did not appear to be significantly activated. These conclusions are supported by antiphosphotyrosine Western blots of specific immunoprecipitates and immune-complex kinase reactions using the artificial substrate enolase. Src and Fyn have been shown to be associated with phosphatidylinositol-3 kinase (PI3K). A time course study of IL-11 stimulated cells reveals increased PI3K activity from antiphosphotyrosine and anti-Yes immunoprecipitated lysates but not anti-Src immunoprecipitated lysates. Src association with PI3K does not change with activation of Src or PI3K by IL-11 but the Yes coprecipitating with PI3K increases with the initial activation. Yes activation appears to coincide with initial PI3K activation. These data identify several new interacting components of the IL-11 response, Src, Yes, and PI3K and suggest that Yes, but not Src, interactions are a part of the PI3K response to IL-11. These conclusions may be applied to other cytokines that utilize the signal transducer gp130.

I 217 SIGNAL TRANSDUCTION OF THROMBIN INVOLVES PHOSPHATIDYLINOSITOL 3 KINASE (PI 3 KINASE) ACTIVATION IN HUMAN GLOMERULAR MESANGIAL CELLS. Goutam Ghosh Choudhury, Giuseppe Grandaliano, Purba Biswas, Bruno Fouqueray and Hanna E. Abboud. Department of Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, TX 78284

Proliferation of mesangial cells is a major histopathological finding in experimental and human glomerular diseases. Intraglomerular thrombosis with thrombin production is also a common feature of severe vascular and proliferative diseases. Thrombin elicits multiple biological effects on a variety of cells. We have recently shown that thrombin is a potent mitogen for human glomerular mesangial cells. In the present study we investigated the role of protein tyrosine phosphorylation in mediating the cellular response to thrombin in human glomerular mesangial cells. Antiphosphotyrosine immunoprecipitation revealed tyrosine phosphorylation of a set of proteins in response to 10 units/ml thrombin in human mesangial cells. Use of tyrosine kinase inhibitors geniestein and herbimycin abolished tyrosine phosphorylation. *In vitro* kinase assay of antiphosphotyrosine immunoprecipitates from thrombin-stimulated mesangial cell lysate showed two tyrosine phosphorylated proteins of 170 kD and 100 kD respectively. The identity of these proteins are currently unknown. Recently it has been shown that activation of a novel PI 3 kinase is a central event in growth factor mediated mitogenesis. In mesangial cells, thrombin stimulated PI 3 kinase activity in a time dependent manner in antiphosphotyrosine immunoprecipitates. The activity peaked at 10 minutes of stimulation and declined at 30 minutes. Use of a monoclonal antibody against c-src demonstrated activation of c-src tyrosine kinase activity in thrombin-treated human mesangial cells. From these data we conclude that thrombin utilizes these two central signal transduction pathways involving tyrosine phosphorylation to elicit its mitogenic effect in human mesangial cell.

I 218 THE ROLE OF PI 3-KINASE IN v-Src-INDUCED CELL TRANSFORMATION AND DIFFERENTIATION, B. Haefner, V. J. Fincham, J. A. Wyke, C. P. Downes* and M. C. Frame. The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, Scotland. *Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland.

Previous results of a number of groups suggest that association with PI 3-kinase plays an important role in v-Src-induced cell transformation and that the SH3 domain of v-Src is involved in mediating this association. Pawson and co-workers have recently shown that the v-Src SH3 domain on its own can bind to PI 3-kinase. We are addressing the role of PI 3-kinase and its cellular phosphorylated lipid products in the transformation of chicken embryo fibroblasts (CEF) and differentiation of PC12 cells by v-Src using temperature-sensitive (*ts*) mutants. One particular transforming mutant of interest is LA32 which has a point mutation in the tyrosine kinase domain and one in the SH3 domain and gives rise to a fusiform morphology in CEF at permissive temperature. We have shown that glutathione S-transferase (GST) fused to the LA32 SH3 domain binds PI 3-kinase from cell extracts as efficiently as GST fused to a wild-type SH3 domain. However, PI 3-kinase activity associated with the whole v-Src LA32 protein immunoprecipitated from CEF is temperature-sensitive, implying an interactive role for these domains in PI 3-kinase activation. Chimaeric proteins between wild-type and LA32 tyrosine kinase and SH3 domains, which have been extensively characterized and produce distinctive morphological phenotypes, are being used to establish the role of these domains in PI 3-kinase activation. This will aid our understanding of the function of PI 3-kinase in mitogenic and morphological aspects of v-Src signalling. In addition, we have stably transfected PC12 cells with *ts* v-Src mutants to study the role of v-Src-associated PI 3-kinase activity in PC12 cell differentiation.

I 219 CLONING OF HUMAN AND MURINE HOMOLOGS OF THE DROSOPHILA POLO SERINE-THREONINE KINASE, **Ryoji Hamanaka[1], Sharon Maloid[2], Mark R. Smith[2], Cathy D. O'Connell[2], Dan L. Longo[1] and Douglas K. Ferris[2]** [1]Laboratory of Leukocyte Biology, Biological Response Modifiers Program, Division of Cancer Treatment, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick MD 21702, USA [2]Biological Carcinogenesis and Development Program; Program Resources/Dyncorp Inc., National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702, USA

We have cloned both human and murine cDNAs that are homologous to the *Drosophila* serine/threonine *polo* kinase and the recently cloned murine *polo*-related kinase (PLK). Both the human and murine clones are about 2.1 Kb with open reading frames of 1.8 Kb, encoding proteins of 603 amino acids with a predicted size of 66 kd and an apparent size of 67 kd by SDS PAGE analysis. During embryonic development of the mouse, the mRNA was expressed in all tissues examined, while in adult tissues, expression was limited to thymus and ovaries. All cell lines examined also expressed mRNAs of similar size. Microinjection of *in vitro* transcribed sense mRNA into serum-starved murine NIH3T3 cells induced tritiated thymidine incorporation while microinjection of antisense RNA into growing NIH3T3 cells blocked tritiated thymidine incorporation. When PC12 rat cells were induced to differentiate with nerve growth factor (NGF), gene expression of PLK was greatly reduced. Together these results suggest that PLK expression is restricted to, and is perhaps required by, proliferating cells.

I 220 SIGNAL TRANSDUCTION THROUGH PDGF α- AND β-RECEPTORS; COMPARISON OF PDGF-INDUCED PROTEIN PHOSPHORYLATION, AS REVEALED BY HIGH-RESOLUTION TWO-DIMENSIONAL POLY-ACRYLAMIDE GEL ELECTROPHORESIS

Klaus Hansen, Lars Rönnstrand, Lena Claesson-Welsh and Carl-Henrik Heldin; Ludwig Institute for Cancer Research, Box 595, S-751 24 Uppsala, Sweden.

Platelet-derived growth factor (PDGF) exerts its cellular effects by binding to and activating two structurally similar tyrosine kinase receptors. The PDGF β-receptor mediates stimulation of cell growth as well as motility. The PDGF α-receptor also mediates stimulation of cell growth, however, its effect on migration is cell type specific; the α-receptor in fact inhibits chemotaxis in certain cell types, like human fibroblasts.
In order to investigate the molecular mechanism responsible for the similarities and differences in effects, we have compared the protein phosphorylation patterns induced by PDGF in transfected PAE cells (porcine aortic endothelial cells) expressing either the PDGF α- or β-receptor, by high-resolution two-dimensional gel electrophoresis. Many protein substrates that are phosphorylated by both α- and β-receptors were identified, including $PLC_{\gamma 1}$, RasGAP and Nck. The band 4.1 protein radixin is like Nck, among the more prominent tyrosine phosphorylated proteins after PDGF stimulation of either α- or β-receptors, while phosphorylation of another band 4.1 protein, ezrin, is unaffected by PDGF stimulation. Besides the already characterized substrates a protein around 42 kDa, different from Erk-1 and -2, is shown to be phosphorylated on tyrosine residues after stimulation of the PDGF receptors.
Certain substrates were phosphorylated only after stimulation of the PDGF β-receptor, e.g. a 130 kDa protein different from $p125^{Fak}$, RasGAP or $PLC_{\gamma 1}$. Furthermore, the β-receptor induces phosphorylation of 81 kDa (different from radixin) and 68 kDa proteins, which are not observed in the case of the α-receptor. So far, we have not been able to identify any PDGF α-receptor specific substrates by employing this high-resolution two-dimensional polyacrylamide gel electrophoresis system.

I 221 ANALYSIS OF THE ROLE OF Csk IN B CELL ANTIGEN RECEPTOR SIGNALING.

Akiko Hata, Hisataka Sabe, Tomohiro Kurosaki#, Minoru Takata# and Hidesaburo Hanafusa, Laboratory of Molecular Oncology, The Rockefeller University, New York, NY10021 and #Department of Cardiovascular Molecular Biology, Lederle Laboratories, Pearl River, NY10965.

In B lymphocytes, antigen stimulation via the B cell antigen receptor (BCR) rapidly causes protein tyrosine phosphorylation, increase of phosphatidylinositol (PtdIns) turnover, and elevation of cytoplasmic calcium. Some of the Src family tyrosine kinases, such as $p53/56^{lyn}$, $p55^{blk}$, and $p59^{fyn}$, are known to physically associate with the BCR complex and are activated by BCR stimulation. $p50^{csk}$ (C-terminal Src kinase) has been identified as a tyrosine kinase which phosphorylates the C-terminal tyrosine residue of the Src family kinases, resulting in down-regulation of their kinase activities. In this study, we made Csk-negative chicken DT40 B cell lines by the targeted disruption of *csk* loci to analyze the role of Csk in BCR signaling. The disruption of *csk* caused activation of a Src family kinase, Lyn. However, BCR stimulation was still necessary to evoke Ca^{2+} influx, PtdIns breakdown, and cellular tyrosine phosphorylation other than autophosphorylation of Lyn in the Csk-negative cells. These results show that the activation of a Src family kinase is not sufficient to induce the signals which are triggered by BCR stimulation. We suggest that, in addition to the activation of Lyn, further mechanisms are necessary to couple the activated Lyn with the BCR complex and induce the cascade of intracellular events in B lymphocytes.

I 222 GENETIC ANALYSIS OF CONVERGING *HEDGEHOG* AND *WINGLESS* SIGNALLING PATHWAYS IN THE DROSOPHILA EMBRYO, Joan E. Hooper, Department of Cellular and Structural Biology, University of Colorado Health Sciences Center, Denver, CO 80262

Two signal transduction pathways define the anterior side of the parasegment border in 3-5 hour Drosophila embryos. Wingless protein, secreted by a row of cells anterior to the parasegment border, remains local. In its autocrine role it instructs cells anterior to the parasegment border to maintain expression of a group of genes, including the *wingless* gene. The *zw3* gene encodes a serine/threonine kinase that participates in the paracrine action of the Wingless signal, maintaining expression of a set of genes in cells posterior to the parasegment border (Siegfried et al., Development 71,1167). This kinase has no role in the autocrine requirement for the Wingless signal. Hedgehog protein, presented by cells posterior to the parasegment border, instructs adjacent cells to maintain expression of a set of genes including the *wingless* gene. Transduction of and/or response to the Hedgehog signal requires function of the *fused* serine/threonine kinase, the *ciD* zinc finger protein, and the *smoothened* gene product. In the *patched* mutant background the Hedgehog signal is no longer required for *wingless* expression; only the Wingless signal is required for *wingless* expression. Since *fused, ciD* and *smoothened* are required for *wingless* expression in the *patched* mutant background, they must function in the signal transduction process downstream of where the Wingless and Hedgehog signals converge.

I 223 CHARACTERIZATION OF RAT PLC-γ1 EXPRESSED IN BACULOVIRUS-INFECTED INSECT CELLS, Debra Horstman and Graham Carpenter, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146

Rat phospholipase C-γ1 (PLC-γ1) was expressed as a histidine-tagged fusion protein in baculovirus-infected insect cells. When a multiplicity of infection of 10 was used and the samples were harvested 48 hours of infection, recombinant PLC-γ1 represented approximately 10% of total insect cell protein. The fusion protein was purified to >85% homogeneity by nickel affinity chromatography. Recombinant enzyme activity was measured using a Triton X-100/PIP_2 mixed micelle assay. The specific activity of the purified recombinant PLC-γ1 was 10umole/min/mg, a value which closely agrees with that reported for purified bovine brain PLC-γ1. In addition, the *src* homology (SH) domains of PLC-γ1 were expressed as a histidine-tagged fusion protein in the same system. Both the full length recombinant PLC-γ1 and its SH domains were tyrosine phosphorylated by immunopurified epidermal growth factor receptor isolated from A-431 cells. These data indicate that using a baculovirus expression system, recombinant PLC-γ1 can be purified in large quantities in a conformationally active form. (supported by NIHCA43720 and HD07043)

I 224 THE PROTO-ONCOGENE C-*SEA* ENCODES A TRANSMEMBRANE PROTEIN TYROSINE KINASE RELATED TO THE MET/HGF/SF RECEPTOR, Janice L. Huff, Mary Anne Jelinek and J. Thomas Parsons, Department of Microbiology, University of Virginia School of Medicine, Charlottesville, VA 22908.

C-*sea* is the cellular homologue of the avian erythroblastosis virus S13-encoded oncogene v-*sea*. We have isolated and determined the nucleotide sequence of overlapping chicken cDNA and genomic clones which encode the putative c-*sea* proto-oncogene product. The predicted reading frame encodes a 1404 amino acid polypeptide which has the structure of a receptor-like protein tyrosine kinase and exhibits sequence similarity with the Met/Hepatocyte growth factor/Scatter factor receptor. The cytoplasmic tyrosine kinase domain of Sea exhibits 70% amino acid identity with Met. The extracellular domain has an overall lower homology (28%), however there is a notable conservation of cysteine residues, as well as the presence of a putative proteolytic cleavage sequence, the latter of which renders the Met protein with an α, β heterodimeric structure. Analysis of the Sea protein expressed in NIH 3T3 cells from a full-length cDNA revealed a similar pattern of proteolytic processing. Thus Sea and Met represent members of a structurally distinctive family of receptor protein tyrosine kinases. The similarity in the overall topology of the extracellular domain of Sea and Met may reflect the binding of a structurally similar or related ligand. We are currently pursuing the identification of the Sea ligand using both *in vitro* binding and biological assay systems. We have constructed a fusion protein consisting of the extracellular domain of the Sea protein linked to the hinge and constant regions of the human IgGγ1 molecule. The resulting protein contains the signal peptide sequence of the SEA receptor, while lacking the transmembrane hydrophobic amino acids and is therefore secreted from transfected cells. As the affinity of a receptor for its cognate ligand is extremely high, this fusion protein can function essentially as a ligand specific antibody. We are currently using this fusion protein to assay for the presence of ligand in chicken tissues and in various conditioned medias.

I 225 INHIBITION OF DIVERGENT βγ-DEPENDENT PATHWAYS EMPLOYING THE βγ-BINDING DOMAIN DERIVED FROM THE β-ADRENERGIC RECEPTOR KINASE, J. Inglese, L. M. Luttrell, I. Boekhoeff, S. Schleicher, W. J. Koch, H. Breer and R. J. Lefkowitz, Howard Hughes Medical Inst., Duke University Medical Ctr., Durham, NC 27710 and Universitat at Hohenheim, Stuttgärt, D-70593.

The β-adrenergic receptor kinases engage in protein-protein interactions with the βγ-subunits of heterotrimeric G proteins which enhance the activity of the kinase by targeting to the membrane-embedded receptor substrate. We have mapped the βARK1 βγ-binding domain to a region in the carboxyl terminus. When expressed as a GST or His6-fusion protein this sequence binds $G_{\beta\gamma}$. These fusion proteins and smaller peptides derived therefrom have been employed to study several signaling pathways known or postulated to be βγ-dependent. In one case, the His_6-βγ-binding domain, purified as a recombinant 30kDa protein from *E. coli*, was introduced into streptolysin O-permeabilized HEK-293. In these cells the proteins antagonized the βγ-mediated α_{2A}-adrenergic receptor stimulation of type II adenylyl cyclase (IC_{50} ~10μM), presumably by sequestering released βγ. In another case, a peptide derived from the βγ-binding domain of βARK2 was used to demonstrate the βγ-dependence of odorant-induced βARK2 targeting to olfactory cila membranes. These domains and peptides were also able to eliminate odorant-induced desensitization and diminish odorant-induced phosphorylation of membrane bound proteins, presumably olfactory receptors. Taken together these findings suggest that $G_{\beta\gamma}$ interacts with a variety of effectors through similar sites and that binding to the various effectors is mutually exclusive.

I 226 *Abstract Withdrawn*

I 227 THE EFFECTS OF bFGF AND aFGF ON BREAST CANCER CELLS

Johnston C.L., Cox H., Coombes R.C. Department of Medical Oncology, Charing Cross and Westminster Medical School, St Dunstans Road, London W6 8RF.

bFGF is present in breast epithelial and myoepithelial cells and remains present in breast cancer cells bFGF receptors are also distributed in epithelial and myoepithelial cells since both these cells types have high affinity binding of 125i-bFGF. Therefore within the breast, release of bFGF from dying cells or by some other mechanism, will effect the behaviour of epithelial and myoepithelial cells. We are interested in looking at the responses of normal breast and breast cancer cells to bFGF to investigate its possible role in breast cancer. We have measured the responses of normal and cancer breast cell lines in terms of growth and membrane ruffling. bFGF acted as a weak mitogen on normal and cancer breast cell lines. None of the normal cell lines responded to bFGF by exhibiting membrane ruffling, however some of the breast cancer cell lines tested did show membrane ruffling in response to bFGF. We have further characterised the membrane ruffling response by using dominant negative FGF receptors.

I 228 A PHOSPHOTYROSINE-INDEPENDENT BINDING MODE OF SH2 DOMAIN, Insil Joung, Indal Park, Tae Ue Kim, and Jeakyoon Shin, Division of Tumor Virology, Dana Farber Cancer Institute, Boston, MA, 02115

A 62 kD protein (p62) that binds specifically to the isolated SH2 domain of p56lck has been identified. p62 is not recognized by anti-phosphotyrosine antibody and is poorly phosphorylated. Association of p62 with the lck SH2 domain is enhanced by dephosphorylation of cell lysate. Furthermore, a mutation of Arg154 to Lys154 in the SH2 domain abolished binding of SH2 domain to phosphotyrosyl proteins without affecting on p62 binding, suggesting that the lck SH2 domain contains a phosphorylation independent binding site in addition to the phosphotyrosyl protein binding pocket. Interestingly p62 binding to the lck SH2 domain is regulated by modification of the Ser59 phosphorylation site in the unique N-terminal region of p56lck and by occupation of phosphotyrosyl protein binding pocket. Phosphorylation-independent binding of unique proteins to SH2 domains derived from different proteins is also characterized. Thus, it is proposed that each SH2 domain has a phosphorylation-independent binding site as well as the conventional phosphotyrosyl protein binding site and that these two binding modes may regulate each other.

I 229 THE ROLE OF IRS-1 AND SHC IN INSULIN-INDUCED SIGNAL TRANSDUCTION

Yasushi Kaburagi¶, Ritsuko Yamamoto-Honda¶, Kazuyuki Tobe¶, Kojiroh Ueki¶,¶¶, Makoto Yachi¶, Yasuo Akanuma¶¶, Yoshio Yazaki¶, David R. Kaplan**, Kazuyoshi Yonezawa*, Akifumi Ando*, Masato Kasuga*, and Takashi Kadowaki¶,¶¶

¶Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, Japan, ¶¶Institute for Diabetes Care and Research, Asahi Life Foundation, Tokyo 100, Japan, *Second Department of Internal Medicine, Kobe University School of Medicine, Kobe 650, Japan, ** National Cancer Institute, Frederick, MD21701

Insulin stimulates the tyrosine phosphorylation of IRS-1 and Shc, and both of them associate with Grb2/ASH, which couples with Sos; a Ras exchange factor (Tobe et al., *J. Biol. Chem.*, 268, 11167, 1993). Ras activation is involved in insulin-stimulated activation of the kinase cascade involving MAP kinase. To examine the role of IRS-1 and Shc in insulin's signalling pathway, we studied the tyrosine phosphorylation of IRS-1 and Shc, the association of Grb2/ASH with these proteins, and MAP kinase activation in CHO cells overexpressing the mutant insulin receptors. In the cells expressing the mutant receptors substituting each of the residues ($Asn^{957}Pro^{958}Glu^{959}Tyr^{960}Leu^{961}Ser^{962}$) in the juxtamembrane region of the receptor by Ala or other amino acids, the substitution of Tyr^{960} by Phe, which impaired the tyrosine phosphorylation of IRS-1, also severely decreased tyrosine phosphorylation of Shc. Moreover, the association of Grb2/ASH with these proteins and MAP kinase activation were decreased. We also found that the substitution of Asn^{957} moderately decreased tyrosine phosphorylation of both IRS-1 and Shc. Cells overexpressing the receptors with the deletion of the COOH-terminal 16, 36, or 64 amino acids exhibited normal receptor autophosphorylation, although the autophosphorylation of the receptors with the deletion of the COOH-terminal 82 residues was markedly diminished. However, none of these COOH-terminal deletions affected tyrosine phosphorylation of IRS-1. In contrast, the tyrosine phosphorylation of Shc was impaired only in the cells expressing the receptors deleting the COOH-terminal 82 residues, in which insulin-induced MAP kinase activation was normal. These data suggest that 1) The juxtamembrane region of the insulin receptor is important for the tyrosine phosphorylation of both IRS-1 and Shc, 2) The tyrosine phosphorylation of IRS-1 appears to be sufficient for the insulin-induced activation of the kinase cascade involving MAP kinase.

I 230 Binding of p91/ISGF3α to a palindromic cis-element mediates IFN-γ induced transcription of genes in the IRF family.

Yuka Kanno and Keiko Ozato. Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development, NIH, Bethesda, MD 20892.

IRF-1, IRF-2, ICSBP and ISGF3γ are members of the interferon regulatory factor family and are induced by IFN-γ. IFN-α/ß induces expression of some members, but not ICSBP. To investigate the IFN-γ mediated induction of the murine ICSBP gene, we have analyzed the promoter region of the gene. A 5 kb of the ICSBP upstream sequence responded to IFN-γ but not appreciably to IFN-α/ß when connected to the bacterial CAT gene. A sequence from -175 to -155 was sufficient to confer IFN-γ induction upon a heterologous promoter. This region does not resemble the canonical interferon stimulated response element (ISRE), and includes a palindromic motif, TTCNNGGAA. Gel mobility shift assays detected an IFN-γ inducible binding activity which was reacted with the anti-p91/ISGF3α antibody. UV crosslinking experiments detected a protein of 90 to 100 kDa in size that contacts the palindromic element. A similar palindromic motif was found in the upstream region of the IRF-1, GBP, MIG and $Fc_\gamma RI$, all of which competed for the factor binding to the palindromic element with different efficiency. Taken together, this palindromic cis-element represents a new class of interferon response element that is capable of conferring IFN-γ induction through the binding of p91/ISGF3α.

I 231 AMPLIFICATION AND OVEREXPRESSION OF THE K-*sam* GENE IN GASTRIC CANCER

Masaru Katoh, Yutaka Hattori, Hiroshi Itoh, Tatsuya Kishi, Hideshi Ishii, Hiromi Sakamoto, Teruhiko Yoshida, Takashi Sugimura and Masaaki Terada. Genetics Division, National Cancer Center Research Institute, Tokyo, Japan

Receptor tyrosine kinase plays a key role in cell growth regulation as well as in human carcinogenesis. The K-*sam* gene is amplified preferentially in undifferentiated types of gastric cancer, and belongs to the gene family of the fibroblast growth factor receptor.

We have isolated four types of K-*sam* cDNAs, which are apparently produced by alternative splicing, from various human tissues and cell lines. Some variant K-*sam* cDNA of keratinocyte growth factor receptor type with a truncated carboxy terminus corresponded to a major transcript in gastric cancer cell line KATO-III cells, and showed higher transforming activity on NIH3T3 cells. Immunohistochemical analysis revealed that gastric cancer with increased amounts of K-*sam* products showed poorer prognosis. Amplification and overexpression of the K-*sam* gene is implicated in the development and the extension of human gastric cancer.

I 232 CELL SURFACE DETERMINATION OF TYPE I INTERFERON BIOLOGICAL ACTIVITY, Susan B. Klein, Osman N. Ozes, Zvi Reiter and Milton W. Taylor, Department of Biology, Indiana University, Bloomington, IN 47405

The type I interferons (IFN) include 16 genetically different natural IFN-α's, IFN-β, and a novel recombinant IFN, r-metIFN-con1 (Amgen Corp.). The biological specific activity (SA) of r-metIFN-con1 (3 X 10^9 units/mg) was shown to be more than one log greater than that of IFN-α2a and IFN-α2b (2 X 10^8 units/mg) in anti-viral response, anti-proliferative response, stimulation of NK cytotoxicity, and induction of interferon stimulated gene (ISG) transcript accumulation. In each case, equal units of IFN/ml induced equivalent biological responses; 20 times more molecules/ml of IFN-α were required to induce the same response. The difference in activity was consistent in the human cell lines ME180, Daudi, and ESKOL as well as 11 non-human cell lines. The cross-species reactivity range was identical to that of IFN-α2b. These results suggest that the determination of SA precedes the cytosolic activation of the ISGF3 subunits, and that the mechanism persists throughout the responsive species. Binding characteristics of r-metINFN-con1 and IFN-α2(a or b) were determined by ^{125}I-IFN binding isotherms and Scatchard analysis. Each type I IFN yielded a biphasic Scatchard plot which deconvoluted into two sites with a two log difference between high and low affinity dissociation constants (k_d). The number of sites bound at minimal effective concentrations was assessed by substitution of the empiracle values for the number of

IFN Subtype	k_d Receptor	k_d 2° Site	#R	#2° Sites	#Bound R	# Bound 2° Sites
IFN-α	3.54×10^{-11} M	2.4×10^{-9} M	843	40885	5.91	4.22
r-con1	0.77×10^{-11} M	1.6×10^{-10} M	564	61013	17.7	9.53

sites (n) and the k_d. IFN was delivered directly to the cell membrane in phosphatidylcholine / phosphatidylserine (7:3) liposomes. Unlike IFN-γ, the liposome delivered IFN-α effected an antiproliferative response after less than 30 minutes of treatment at doses 7.8 times lower than free IFN. The response was blocked by either anti-IFN-α or anti-IFN receptor antibody. From these data we hypothesize that the biological activity depends cell surface phenomena.

I 233 THE CARBOXYL TERMINUS OF A G PROTEIN-COUPLED RECEPTOR KINASE ACTS AS A CELLULAR Gβγ-ANTAGONIST, Walter J. Koch, Brian E. Hawes, James Inglese, Louis M. Luttrell, Neil J. Freedman and Robert J. Lefkowitz, Howard Hughes Medical Institute and the Departments of Medicine and Biochemistry, Duke University Medical Center, Box 3821, Durham, NC 27710.

The βγ subunits (Gβγ) of heterotrimeric guanine nucleotide-binding (G) proteins modulate the activity of several signal transducing effector molecules including phospholipase C (PLC) and G protein-coupled receptor kinases. Gβγ binds to the carboxyl terminus of the β-adrenergic receptor kinase (βARK), and regulates its activity by targetting to its membrane bound receptor substrate. Previous *in vitro* experiments have shown that βARK carboxyl terminal peptides can inhibit Gβγ-activation of βARK1. Here we report intact cell data showing that co-expression in COS 7 cells of the βARK1 carboxyl terminus, containing the Gβγ-binding domain, with the β_2-adrenergic receptor results in attenuation of agonist-induced receptor phosphorylation. This data supports the hypothesis that, in a cellular setting, Gβγ does regulate βARK activity. To investigate the effect of such a Gβγ-binding domain on heterologous Gβγ interactions, we co-expressed in COS 7 cells various PLC-coupled receptors with the carboxyl terminus of βARK1. Phosphoinositol hydrolysis in response to activation of receptors which stimulate PLC via Giβγ (α_2-adrenergic (AR) and M2-muscarinic cholinergic (AChR)) was markedly inhibited by the co-expressed βARK1 polypeptide whereas that mediated by Gqα subunits (α_1-AR and M1-AChR) was unaffected. Moreover, inhibition of adenylate cyclase produced by α_2-AR stimulation (a Giα-mediated process) was unaffected, indicating that the βARK1 polypeptide provides a useful tool for distinguishing between Gα and Gβγ pathways.

I 234 **Rapid Activation of DNA Binding Proteins in Response to Multiple Cytokines.**

P. Lamb, L.V Kessler, C. Suto, M. Seidel, R.B. Stein and J. Rosen, Ligand Pharmaceuticals, 9393 Towne Centre Drive, San Diego, CA 92121.

Many cytokines and growth factors trigger rapid changes in gene expression upon binding to their receptors. In many cases the mechanism by which these changes are affected is unknown. We show that interleukin 2 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), leukemia inhibitory factor (LIF) and granulocyte-macrophage colony stimulating factor (GM-CSF) treatment of cells causes rapid activation of DNA binding activities that recognize a DNA sequence element previously implicated in regulation of gene expression by interferon γ (IFNγ). The properties of the IL-4, IL-6 and GM-CSF induced complexes were compared with the recently characterized protein p91 that is activated by IFNγ. Complexes induced by IL-4, IL-6 and GM-CSF are distinguishable from the the IFNγ induced complex on the basis of their mobility in polyacrylamide gels, sequence preferences, sensitivity to NaCl and lack of reactivity with an anti-p91 antiserum. The IL-4 and GM-CSF induced complexes react with anti-phosphotyrosine antibodies demonstrating the importance of phosphotyrosine containing proteins in these DNA binding complexes. Further characterization of these complexes will be presented. Transcriptional activation of a reporter gene linked to a synthetic IFNγ-responsive promoter is observed in response to these cytokines. These data suggest a pathway by which cytokines induce rapid changes in gene expression.

I 235 Signal Transduction Mechanisms Utilized by Lymphoid Cells After Engagement of the Early Activation Antigen CD69, by Steven D. Levin, Fred Ramsdell, Katherine Hjerrild, and Steven F. Ziegler, Immunex Corporation, 51 University Street, Seattle, WA 98101.

Expression of CD69 is among the earliest consequences of T cell activation. Triggering of the T cell antigen receptor and activators of protein kinase C induce a 10-50 fold induction in the accumulation of CD69 transcripts that reaches a maxima within two hours and returns to baseline levels by 24 hours. Surface expression of the protein is detectable within one hour and persists for at least 72 hours after stimulation. While no well-defined function has yet been ascribed to CD69, stimulation of T cells with antibodies to CD69 plus suboptimal doses of anti-CD3 antibodies results in a more vigorous response than with anti-CD3 antibodies alone, indicating CD69 is a costimulatory molecule. We have used a re-directed killing assay in the transformed human NK cell line YT to assess the mechanisms involved in the transduction of CD69 mediated signals. Preliminary results suggest that propagation of CD69-mediated signals may depend on the phosphorylation of one or more serine residues in the cytoplasmic domain of the CD69 protein. To determine the significance of this serine phosphorylation, we have mutated each of the 8 serine residues in the cytoplasmic domain of CD69 and have introduced these mutant forms into YT cells. These mutants will be assessed for their ability to function in a re-directed killing assay, and thus should provide useful information regarding the mechanisms of signal transduction employed by the CD69 molecule.

I 236 RAPID CYTOKINE AND GROWTH FACTOR SIGNALING THROUGH TYROSINE PHOSPHORYLATION OF A FAMILY OF CYTOPLASMIC TRANSCRIPTION FACTORS, David E. Levy and Olli Silvennoinen, Department of Pathology, NYU School of Medicine, New York, NY 10016

Immediate early gene transcription is crucial to the action of polypeptide growth factors and cytokines. For interferons, transcriptional activation is mediated by tyrosine phosphorylation of the latent, cytoplasmic transcription factor ISGF3. We report that growth factors and cytokines use a similar pathway: the 91 kDa subunit of ISGF3 was activated through tyrosine phosphorylation in response to epidermal growth factor, platelet-derived growth factor, and colony stimulating factor 1, but not to insulin or fibroblast growth factors. Tyrosine phosphorylation led to nuclear accumulation and stimulation of DNA-binding activity. Activation required the major sites for autophosphorylation on the EGF receptor, suggesting assembly of a multimeric signaling complex of tyrosine-phosphorylated and SH2-domain-containing proteins. Responses in dominant inhibitory Ras mutant cells indicated that this process is independent of the normal functioning of Ras, defining a novel pathway for direct growth factor signaling from the cell surface to the nucleus. The cytokines IL2, IL3, and erythropoietin caused tyrosine phosphorylation and nuclear accumulation of additional proteins which share homology with ISGF3 p91 and constitute a multigene family of signaling transcription factors.

IFNα/β, IFNγ, EGF, and PDGF signal through independent cell surface receptors to activate overlapping components of the ISGF3 family of transcription factors. Genetic complementation studies with IFN resistant cell lines implicated the tyrosine kinase Tyk2 in IFNα signaling and, more recently, Jak2 in IFNγ signaling. We present biochemical evidence for Jak1 and Jak2 tyrosine kinase involvement in IFNα and IFNγ signal transduction as well as in EGF and PDGF signaling. Both Jak1 and Jak2 were activated by IFNγ, whereas IFNα activated only Jak1. Moreover, overexpression of either Jak1 or Jak2 stimulated p91 DNA-binding activity. Overexpressed Jak1 was also an efficient activator of endogenous Jak2, suggesting that a Jak kinase cascade transduces signals from cytokine receptors through tyrosine phosphorylated transcription factors to the nucleus.

I 237 ANTIBODY CROSS-LINKING OF β_2 INTEGRINS INDUCES INCREASE OF INTRACELLULAR FREE CALCIUM ION CONCENTRATION VIA HERBIMYCIN A-INSENSITIVE PATHWAYS IN HUMAN NEUTROPHILS, Klaus Ley, Barbara Walzog, Andreas Zakrzewicz, Peter Gaehtgens, Roland Seifert, Departments of Physiology and Pharmacology, Freie Univ. Berlin D-14195, Germany

Human neutrophils (PMN) exert most of their physiological functions while adherent to surfaces rather than in suspension. PMN adhesion is largely dependent on the function of the β_2 integrins, CD11a,b,c/CD18. We mimicked engagement of β_2 integrins by antibody cross-linking of CD18 on isolated human PMN using both intact mAb and $F(ab')_2$ fragments. Within seconds of CD18 cross-linking, we observed a significant, transient rise of intracellular free Ca^{2+} concentration by 200-300 nM, which was largely due to Ca^{2+} mobilization from intracellular stores. The Ca^{2+} signal was blocked after pretreatment with PMA, an activator of protein kinase C, but not with herbimycin A, a potent inhibitor of tyrosine kinases. In addition to the rise of intracellular free Ca^{2+} concentration, CD18 cross-linking induced exocytosis of azurophilic granules (release of 26% of total PMN elastase), which was significantly inhibited by herbimycin A. Moreover, exocytosis of specific granules (2.2-fold up-regulation of CD18 antigen) and significant down-regulation of surface expression of the granulocyte adhesion molecule L-selectin were induced. CD18 cross-linking by soluble antibodies did not induce superoxide production, but PMN bound to immoblized mAb against CD18 released significant amounts of superoxide. In contrast to recent findings in T-lymphocytes (Kanner at al., PNAS 90:7099, 1993), initial signaling through β_2 integrins does not appear to be mediated by a phospholipase C isoform activated by tyrosine phosphorylation, because the Ca^{2+} signal was not altered by herbimycin A. However, more complex cellular responses including exocytosis were found to require tyrosine phosphorylation. Engagement of β_2 integrins provides an important stimulatory signal to PMN which is likely to augment activation by soluble inflammatory mediators.

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I 238 **THE HUMAN ST5 GENE PRODUCTS: AN SH3 BINDING PROTEIN AND A TRUNCATED FORM DOWN-REGULATED IN CERVICAL CARCINOMAS**, Jack H. Lichy, Molecular Diagnostics Laboratory, Department of Cellular Pathology, Armed Forces Institute of Pathology, Washington, D.C. 20306-6000.

The ST5 gene, originally identified as a differentially expressed gene in a system of tumorigenic and non-tumorigenic HeLa/fibroblast somatic cell hybrids, is expressed as three distinct RNA species having lengths of 4.6, 3.1, and 2.8 kb. Whereas non-tumorigenic hybrids express all three species, expression of the 2.8 kb RNA is virtually undetectable in their tumorigenic derivatives. This message is also markedly down regulated in a subset of cervical carcinoma cell lines examined. Full length ST5 cDNA clones have been isolated. The 4.6 and 3.1 kb mRNAs are alternatively spliced products, sharing common 5' and 3' ends but differing in that the 3.1 kb species lacks 1660 nt of internal sequence. The 2.8 kb mRNA is transcribed from a distinct promoter located within an intron of the ST5 gene. Transient expression studies demonstrated specific expression of this promoter in the non-tumorigenic cell lines and mapped the sequence elements responsible for specific expression to an enhancer located 1500-1800 nt upstream of the splice junction. The 4.6 kb cDNA contains an open reading frame encoding a 126 kDa protein. The predicted amino acid sequence of this protein reveals two proline rich regions, PR1 and PR2, which contain a total of seven potential SH3 binding motifs and are both encoded by the 1660 nt unique to the 4.6 kb mRNA. Expressed as GST fusion proteins, PR1 bound weakly and PR2 strongly to a labeled c-*abl* SH3 domain probe. The protein sequence also contains 21 repeats of the motif S/T-P, including five occurrences of the expanded motif P-X-S/T-P, suggesting that ST5 may be a target of regulation by the MAP kinase cascade.

I 239 CHARACTERIZATION OF A NOVEL SH2 DOMAIN IN BAND 3, Philip S. Low[1], Chang Cheng Wang[1], and Marietta L. Harrison[2], Department of Chemistry[1] and Department of Medicinal Chemistry and Pharmacognosy[2], Purdue University, West Lafayette, IN 47907

Red cell tyrosine kinases have been shown to phosphorylate band 3 and thereby control the association of several glycolytic enzymes with an inhibitory binding site at the N-terminus of band 3 (1-3). Recent data have revealed that one of the kinases responsible for band 3 phosphorylation in vivo is $p72^{syk}$, with possible participation also by $p56/53^{lyn}$ (4). Since $p72^{syk}$ was found to immunoprecipitate with band 3, we examined its possible site of interaction with the anion transporter. Sequence analyses indicate that band 3 contains the conserved regions of an SH2 domain, however the homologous sequences comprise cytoplasmic loops separated by nonhomologous membrane-spanning sequences. Binding studies show that $p56/53^{lyn}$ associates with band 3 only after tyrosine phosphorylation (K_D ~10^{-6}M) and that this interaction is blocked by antibodies to the cytoplasmic loops on band 3. Similar results are seen with the isolated, tyrosine phosphorylated cytoplasmic domain of band 3. These data suggest that band 3 may contain a novel, noncontiguous SH2 domain at its cytoplasmic surface that can associate with the tyrosine phosphorylated states of its own cytoplasmic domain and with a kinase possibly involved in its phosphorylation.

1. Low, P.S., Allen, D.P., Zioncheck, T.F., Chari, P., Willardson, B.M., Geahlen, R.L., and Harrison, M.L. (1987) *J. Biol. Chem.* 262, 4592-4596
2. Harrison, M.L., Rathinavelu, P., Arese, P., Geahlen, R.L., and Low, P.S. (1991) *J. Biol. Chem.* 266, 4106-4111
3. Low, P.S., Rathinavelu, P., and Harrison, M.L. (1993) *J. Biol. Chem.* 268, 14627-14631
4. Harrison, M.L., Isaacson, C.C., Burg, D.L., Geahlen,R.L., and Low, P.S. (1993) *JBC*, in press.

I 240 SRC HOMOLOGY 2 DOMAINS OF PROTEIN TYROSINE PHOSPHATASE (SH-PTP2) BIND TO BOTH INSULIN RECEPTOR AND INSULIN RECEPTOR SUBSTRATE-1 (IRS-1) via YXXM motifs *in vitro*. Hiroshi Maegawa, Satoshi Ugi, Masaaki Hasegawa, Rie Tachikawa-Ide, Masanori Iwanishi, Atsunori Kashiwagi, Jerrold M. Olefsky and Yukio Shigeta, Third Department of Medicine, Shiga University of Medical Science, Ohtsu, Shiga, JAPAN and Department of Medicine. University of California, La Jolla, CA 92161

To clarify the role of protein tyrosine phosphatase (PTPase) containing Src homology 2 (SH2) regions on insulin signaling, we investigated the interaction among insulin receptor, SH2 domains of SH-PTP2 and insulin receptor substrate-1 (IRS-1) fusion proteins (amino-portion; amino acid 433-764 IRS-1N and mid to carboxyl-portion; amino acid 903-1138, IRS-1M) coupled to glutathione-S-transferase (GST). GST-SH2 protein of SH-PTP2 was phosphorylated by insulin receptor kinase and the wild type insulin receptor, but neither the carboxyl-terminal truncated nor mutated (Y/F2) insulin receptor was co-immunoprecipitated with GST-SH2 protein by anti-GST antiserum (α GST), suggesting that SH2 domains of SH-PTP2 might bind to insulin receptor and putative binding site of SH2 domain might be at Y1322 on the carboxyl-terminus of insulin receptors. Furthermore, the GST-uncoupled SH2 protein was also co-immunoprecipitated with phosphorylated GST-IRS-1M, but not with phosphorylated GST-IRS-1N by α GST, whereas each GST-IRS-1 fusion protein (N and M) contains several YXXM motifs. These results indicate that SH2 domains of SH-PTP2 can directly associate with the Y^{1322}XXM motif on the carboxyl-terminus of insulin receptors and also can bind to same YXXM motifs on the mid to carboxyl-portion, but not on the amino-portion of IRS-1 *in vitro*. Biological significance of these association of SH-PTP2 with either insulin receptor or IRS-1 is under investigation.

I 241 IDENTIFICATION, PURIFICATION AND MOLECULAR CLONING OF BLK, LYN AND FYN(T) SH2 BINDING PROTEINS FROM B LYMPHOCYTES, Sami N. Malek and Stephen Desiderio, Department of Molecular Biology and Genetics and the Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.

Blk, Lyn and Fyn(T) are tyrosine kinases of the Src family that have been implicated in signalling through the B cell antigen receptor (sIg). Using an in vitro assay for phosphoprotein binding to GST-SH2 fusion proteins, we found that the SH2 domains of Blk, Lyn and Fyn(T), when tested under conditions that favor high-affinity interactions, preferentially bind distinct sets of phosphoproteins from B cells. A subset of the proteins recovered from a Blk SH2 affinity matrix are substrates for sIg-activated tyrosine kinase(s). [Malek, S. N. and Desiderio, S., J. Biol. Chem., in press.] By SH2 affinity chromatography, we have obtained sufficient amounts of selected Blk SH2 binding proteins for isolation of peptides and determination of amino acid sequence. We have obtained sequences of peptides from five Blk SH2 ligands: pp50, pp72-76, pp90, pp130 and pp150. An oligonucleotide based on peptide sequence from pp90, which preferentially binds the Blk SH2 domain, was used to isolate a partial cDNA from spleen; as this cDNA encodes several other peptides from pp90, it appears to represent the pp90 transcript. The predicted, partial sequence of pp90 has no known homologues. Degenerate, synthetic oligonucleotides, based on peptide sequences from pp76, were used to amplify a partial cDNA. This probe was used to isolate a cDNA clone containing an open reading frame of 749 codons; by sequence, this clone appears to encode a serine/threonine kinase. The in vitro translation product of this cDNA clone migrates anomalously slowly on SDS-PAGE gels. Antibodies against this putative kinase detect a major species with an apparent size of 130 kDa and a minor species with an apparent size of 76 kDa in B cell lysates and among proteins eluted from the Blk SH2 affinity matrix, suggesting that the kinase interacts, directly or indirectly, with the Blk SH2 domain.

I 242 CHARACTERIZATION OF THE TEC PROTEIN-TYROSINE KINASE IN THE MURINE HEMATOPOIETIC SYSTEM, Hiroyuki MANO, Ken SATO and Hisamaru HIRAI, Department of Molecular Biology, Jichi Medical School, Yakushiji 3311-1, Kawachi-gun, Tochigi-ken 329-04, JAPAN

Tec has been introduced as a novel member of non-receptor-type protein-tyrosine kinase. The Tec kinase lacks an N-terminal myristylation signal or a C-terminal phosphotyrosine acceptor site. Recently, several Tec-related tyrosine kinases have been identified, one of which is presumed to be the responsible gene for the X-chromosome-linked agammaglobulinemia. All members of the Tec family are abundantly expressed in hematopoietic tissues. Thus, it has been an intriguing issue to investigate the in vivo role of Tec in myeloid cells. Here we show that alternative splicing of the Tec message can alter N-, C-termini and SH-3 domain of the Tec protein. We also demonstrate that, by using anti-Tec serum, stimulation with IL-3 can induce phosphorylation and kinase activity of the Tec protein-tyrosine kinase in a murine myeloid cell line. IL-3-stimulation can also induce physical association of Tec with other phosphoproteins. These results suggest that Tec is a good candidate for a member of signaling complexes of IL-3. Furthermore, we have identified cellular proteins which can associate Tec either in an IL-3-dependent or an IL-3-independent manner.

I 243 THE SH2 DOMAIN PROTEIN, GRB-7, IS AMPLIFIED, OVEREXPRESSED, AND IN A TIGHT COMPLEX WITH HER2 IN BREAST CANCER, B. Margolis, D. Stein, J. Wu, S. Fuqua[3], C. Roonprapunt, J. J. Moskow[2], A.M. Buchberg[2], and C. K. Osborne[3], Department of Pharmacology, New York University Medical Center, [2]Jefferson Cancer Institute, Thomas Jefferson Medical College, [3]Divison of Medical Oncology, University of Texas at San Antonio.

SH2 domain proteins are important members of the tyrosine kinase signaling cascade. We have been cloning SH2 domain proteins by bacterial expression cloning based on their ability to bind phosphotyrosine containing sequences within the carboxyterminus of the EGF-Receptor. One of these newly cloned SH2 domain proteins, GRB-7, was mapped on mouse chromosome 11 to a region which also contains the tyrosine kinase receptor c-erbB2/HER2. Because this receptor is often amplified in breast cancer, we examined the amplification and expression of GRB-7 in breast cancer cell lines. GRB-7 is amplified and overexpressed in concert with HER2 in several breast cancer cell lines and this overexpression is also observed in tissue obtained from breast cancer patients. GRB-7 binds tightly to HER2 via its SH2 domain such that almost all of the tyrosine phosphorylated HER2 in the breast cancer cell line, SKBR-3, is bound to GRB-7. GRB-7 can also bind tyrosine phosphorylated SHC albeit at a lower affinity than GRB2. We have also found that GRB-7 has a strong similarity over more than 300 amino acids to a new gene identified by the C. Elegans Genome Sequencing Project in a region outside of the SH2 domain. Although we do not know the exact signaling pathway on which GRB-7 lies, the presence of evolutionarily conserved domains indicates it is likely to perform a basic signaling function. The fact that GRB-7 and HER2 are both overexpressed and bound tightly together raises the possibility that this signaling pathway is tremendously amplified in certain breast cancers.

I 244 THE BIOCHEMICAL BASIS OF SYNERGY BETWEEN INTERLEUKIN-4 AND INTERLEUKIN-10, Alice L-F. Mui and Atsushi Miyajima, DNAX Research Institute for Molecular and Cellular Biology, Palo Alto, CA 94304

The cytokine Interleukin-3 (IL-3) is an important regulator of hemopoiesis with a wide range of action on cells of all stages of hemopoietic development. One of its activites includes the support of growth of mast cell progenitor and mast cell lines. Interleukin-4 (IL-4) and interleukin-10 (IL-10) are also important hemopoeitic cytokines, however, their range of action are more restricted: unlike IL-3, neither are able to sustain the proliferation of mast cells. The combination of IL-4 and IL-10, however, is able to support growth and replace mast cell requirement for IL-3. To investigate the biochemical basis of this synergy, various intracellular signalling events were examined. All three cytokines rapidly induce tyrosine phosphoryation of many cellular substrates. However, as the pattern of tyrosine phosphorylation was unique to each cytokine and the combination of IL-4 and IL-10 did not result in a pattern resembling that of IL-3 stimulation, the synergy between IL-4 and IL-10 must be downstream of these early protein tyrosine phosphorylations. Downstream signalling events examined include Ras, SHC, Vav, Raf kinase, and MAP kinase activation and fos induction. Although IL-3 clearly stimulated these events, neither IL-4 or IL-10 or the combination of the two appears to do so. IL-3 stimulation also results in the association of phosphatidyl inositol 3' kinase with tyrosine phosphorylated proteins and myc induction. Both IL-4 and IL-10 also induced these events, however the combination did not result in levels above each alone. Thus the synergy between IL-4 and IL-10 may lie outside of all these pathways. Candidate pathways in which the synergy may occur include those which leads to phosphorylation of the retinoblastoma gene product (Rb) since IL-3 or the combination of IL-4 and IL-10 but not IL-4 or IL-10 alone stimulates the phosphorylation of Rb. Studies directed at investigating the events upstream of Rb phosphorylation are underway.

I 245 THE splA TYROSINE KINASE IN *DICTYOSTELIUM* IS REQUIRED FOR SPORE FORMATION, Glen H. Nuckolls and James A. Spudich, Dept. of Biochemistry, Stanford Univ. School of Medicine, Stanford, CA 94305

We have used homologous recombination to disrupt the *splA* gene (formerly called DPYK1), which encodes a developmentally expressed tyrosine kinase in *Dictyostelium discoideum*. Cell lines in which the gene has been disrupted proceeded through the developmental cycle with normal timing and generated fruiting bodies with only slight developmental abnormalities. However, microscopic examination of the spores from these fruiting bodies revealed that they had spontaneously lysed, hence the name *"spore lysis A" (splA)*. The mutant spores exhibited less than 0.5% of the viability of wild type spores treated in a similar manner. Staining of wild type and *splA*$^-$ spores with a fluorescent dye that binds to the spore coat showed bright staining on wild type spores and only weak staining on the mutant spores. Immuno-Western blots indicated that the spore coat component Dd31 was expressed with normal timing in the mutant spores. (Anti-Dd31 antibody provided by W.F. Loomis, UCSD) These data suggest that the mutant cells receive signals that initiate the formation of spores, however the cells lyse prior to the formation of mature spores. The splA kinase may be involved in regulating the expression of other spore coat components, or in the secretion and assembly of these components into a spore coat.

I 246 THE DROSOPHILA HOMOLOG OF ETS-1 FUNCTIONS DOWNSTREAM OF ras THE sevenless SIGNAL TRANSDUCTION CASCADE, Elizabeth M. O'Neill and Gerald M. Rubin, Department of Molecular and Cell Biology, UC Berkeley, Berkeley, CA, 94720

The gene *sevenless* codes for a receptor type tyrosine kinase whose activity is required for proper cell fate specification of the R7 photoreceptor in the Drosophila eye. Ras1 has been shown to function downstream in the signal transduction cascade initiated by ligand-bound Sevenless. Expression of an activated form of Ras1 in a subset of cells in the developing eye causes the differentiation of supernumerary R7 cells, resulting in a rough eye phenotype.

In an attempt to identify genes that might function downstream of Ras1 in this pathway, we screened through a collection of 600 P-element induced embryonic lethal genes for dominant suppressors of the rough eye phenotype caused by misexpression of activated Ras1. We identified several alleles of *pointed*, the Drosophila homolog of the transcription factor ets-1, in this screen. We will present data showing the genetic interactions of *pointed* with other genes in the *sevenless* pathway as well as a phenotypic analysis of the role for *pointed* in other aspects of eye development.

I 247 DRK, A DROSOPHILA SH2-SH3 ADAPTOR PROTEIN IMPLICATED IN COUPLING THE SEV TYROSINE KINASE TO SOS, AN ACTIVATOR OF RAS. J.P. Olivier[1], T. Raabe[3], M. Henkemeyer[1], G. Mbamalu[1], B. Dickson[3], B. Margolis[2], J. Schlessinger[2], E. Hafen[3], and T. Pawson[1] 1) Mt Sinai Hospital, Toronto, Canada M5G 1X5, 2) NYU Medical Center, New York, NY 10016, 3) University of Zurich, CH-8057 Zurich, Switzerland.

Drk/Grb2/Sem-5 encode an adaptor protein comprised of SH3-SH2-SH3 domains. *Drk* maps to polytene chromosome position 50A and the mutations E(*sev*)2B and Su(*sev*^{S11})R1 identified through their genetic interactions with the DER and *Sevenless* receptor tyrosine kinases (RTK). Both mutations carried point mutations within conserved residues of the Drk SH2 domain. P-element rescue experiments using Drk cDNA verified that the pupal lethality of E(*sev*)2B/ Su(*sev*^{S11})R1 compound heterozygotes is complemented by the Drk cDNA.

In vitro binding studies show that GST-Drk fusion protein binds to an activated Torso-Sev chimeric protein through its SH2 domain whereas Drk protein carrying the either the E(sev)2B or Su(*sev*^{S11})mutation in the SH2 domain do not bind.

*Ras*1, a guanine nucleotide binding protein, and *Son of sevenless* (*Sos*), a guanine nucleotide releasing protein are also required for sevenless signaling. To investigate the potential interactions of Drk with downstream molecules *in vitro* binding studies were performed with Drk and Sos proteins. The results indicate that Drk binds specifically to the C-terminal portion of Sos which is proline rich. These results suggest Drk functions as an adaptor molecule by binding to the autophosphorylated RTK via its SH2 domain and to Sos through its SH3 domains thereby coupling RTKs to Ras activation.

I 248 RAS MEDIATES SRC BUT NOT EGF-R TYROSINE KINASE SIGNALING PATHWAYS IN GH4 NEUROENDOCRINE CELLS, Cheryl A. Pickett and Arthur Gutierrez-Hartmann, Department of Medicine and of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver, CO 80262.

Protein tyrosine kinase activation has been implicated as the initial event in mediating the effects of many growth factors on cell growth, differentiation and neoplasia. While many potential signal transducers of tyrosine kinase activation have been identified, the precise functional role of these signal transducers and the interplay between them which can ultimately result in a cell-specific response to a ubiquitous growth factor/receptor remains unclear. We have utilized a transient co-transfection model system in EGF responsive cultured neuroendocrine (GH4 rat pituitary) cells, to investigate the role of p21ras in coupling EGF receptor and v-src tyrosine kinase signals to the activation of a cell specific gene promoter for prolactin (PRL). The GH4 cell is of particular interest because EGF exposure appears to stimulate differentiation and enhance transcription of the cell-specific product (PRL), in contrast to many other cell types wherein EGF exposure produces a mitogenic response. Employing this system, a dose- and time-dependent EGF stimulation of the transfected rPRL promoter luciferase reporter construct was obtained with half-maximal response at 80 pM EGF and a maximal stimulation of 4-10 fold. A similar degree of activation of the rPRL promoter was obtained by co-transfection of a plasmid encoding v-src. Co-transfection of a construct encoding the dominant-negative ras (N17 ras) produced almost complete inhibition of v-src activated rPRL promoter activity. In contrast, EGF stimulated rPRL promoter activity was not inhibited by the N17 ras construct. EGF and v-src were also found to produce large increases in expression of another promoter reporter construct, pc-fos TK luc; and once again EGF stimulated activation was not inhibited by expression of the N17 ras construct, while v-src activation was blocked. Hence, in contrast to data from other cell types, in the GH4 rat pituitary cell, ras is not a primary downstream effector of EGF-mediated control of gene transcription. This lack of ras-mediated signaling appears to be cell specific rather than promoter specific. Thus, it would seem that the EGF receptor-initiated signal in GH4 pituitary cells is preferentially conducted down an alternate (non-ras mediated) pathway, despite the presence of functional ras and its downstream effectors. These observations imply that the machinery available for coupling the EGF-R tyrosine kinase to ras in this neuroendocrine cell differs from that in many other cell lines, emphasizing that cell-specific differences in the response to a given growth factor may depend on the amounts or activities of the various components of the signaling pathway.

I 249 A MULTIFUNCTIONAL DOCKING SITE MEDIATES SIGNALLING AND TRANSFORMATION BY THE HEPATOCYTE GROWTH FACTOR / SCATTER FACTOR (HGF/SF) RECEPTOR FAMILY

C.Ponzetto, A.Bardelli, F.Maina, E.Audero and P.M.Comoglio
Dept. of Biomedical Sciences & Oncology, Turin University School of Medicine, C.so M. D'Azeglio 52, 10126 Turin, Italy

This work reports evidence of the existence in the HGF/SF receptor of a multispecific binding site for several SH2-containing effectors. Signal transduction by the HGF/SF receptor (the transmembrane tyrosine kinase encoded by the *MET* proto-oncogene) is likely to involve the integration of several pathways, given the complex series of biological responses that the ligand evokes in epithelial cells (mitogenesis, motogenesis and morphogenesis). We have previously shown that the HGF/SF receptor binds and activates PI3-kinase and a tyrosine phosphatase and activates a *Ras* nucleotide exchanger. We now show that the bulk of the receptor signalling activity is funneled through the phosphotyrosine pair Y^{*1349}VHVNATY^{*1356}VNV which binds PI3-kinase, PLCγ, GRB-2 and pp60$^{c\text{-}src}$. The interactions of the SH2 domains of all these molecules with phosphopeptides including Y^{*1349} or Y^{*1356} are characterized by fast association and dissociation rates, consistent with the notion of a multifunctional docking site. Mutation of the two tyrosines results in loss of biological function, as shown by the abrogation of the transforming activity in the oncogenic counterpart of the receptor. The *bidentate* motif Y-hydrophobic-X-hydrophobic-$(X)_3$-Y-hydrophobic-N-hydrophobic is conserved the evolutionary related receptors *Sea* and *Ron* and mediates their interaction with the same SH2 domains. We suggest that ligand-induced phosphorylation of the multifunctional docking site is the main transductional switch in the HGF/SF receptor family.

I 250 INTERFERON SIGNALING DEFICIENCY IN HUMAN MELANOMA CELL LINES, Steve Ralph, Bruce Wines, Mike Payne, Irene Hatziniziriou, Rod Devenish and Tony Linnane, Center for Molecular Biology and Medicine, Clayton, Victoria , Australia, 3186.

Clinical therapy of malignant melanomas with interferons produces responses in only a limited number of cases. We developed an in vitro biological assay for testing melanoma cell responses to the interferons (1&2) and have improved our test system by changing to an anti-viral assay, whose advantages we shall describe. The interferon responsiveness of the melanoma cells in the anti-viral assay correlated with the level of change in expression of oligo adenylate synthase and the Mx antigen. A comparison of the cell lines for interferon activated signal transduction revealed that pre-treating responsive cell lines with tyrosine kinase inhibitors produced a dose-dependent inhibition of the ability of interferons to protect cells from viral killing. In addition, the interferon responsiveness of the melanoma cell lines correlated with changes in the observed levels of tyrosine phosphorylated cellular proteins (Mw 80-140Kd). We propose that the lack of interferon response in interferon resistant melanoma cell lines results from a deficiency in the tyrosine kinase signal transduction pathway. No significant differences have yet been found in the levels of interferon binding, receptor numbers, the sequences of the type 1 receptors or levels of TYK-2 mRNA expressed in these cells.

1) Johns,TG, Mackay, IR, Callister, KA et al. 1992 J Natl. Cancer Inst. 84:1185-1190.
2) Loveland, BE, Johns, TG, Mackay, IR et al. 1992 Biochem Intl. 27:501-510.

I 251 INTERACTION OF SHC WITH THE T CELL RECEPTOR ζ CHAIN MAY COUPLE T CELL ACTIVATION TO THE RAS SIGNALING PATHWAY,

Kodimangalam S. Ravichandran, Kyungah K. Lee, Zhou Songyang, Lewis C. Cantley, Paul Burn and Steven J. Burakoff[1]
Division of Pediatric Oncology, Dana-Farber Cancer Institute, and the Department of Pediatrics, Harvard Medical School, Boston, MA 02115.

The *shc* oncogene product is composed of a Src-homology 2 (SH2) domain and a collagen-like region without an obvious catalytic domain. Shc is phosphorylated on tyrosine in cells overexpressing Src-family kinases and upon its phosphorylation it interacts with another adapter protein Grb2. Grb2, in turn, interacts with the GTP/GDP exchange factor for Ras, mSOS. Since Shc functions upstream of Ras and T cell activation involves several Src-family kinases, the role of Shc in T cell receptor (TCR)-mediated Ras activation was examined. Upon crosslinking of the TCR, Shc was phosphorylated on tyrosine and it subsequently interacted with Grb2 and mSOS. In addition, several lines of evidence suggested that Shc, via its SH2 domain interacts directly with the ζ chain of the TCR: (1) Shc SH2 domain precipitated phosphorylated ζ chain from activated T cell lysates; (2) Shc was precipitated from unactivated T cell lysates by a peptide corresponding to the third tyrosine-based activation (TAM) motif in ζ (with the tyrosines phosphorylated), but not by the non-phosphorylated ζ peptide nor by a peptide corresponding to a similar motif in CD3-ε chain; (3) In Jurkat T cells expressing CD16-ζ chimeric molecule (extracellular domain of CD16 and cytoplasmic domain of ζ), activation via CD16 crosslinking led to co-precipitation of Shc with the chimeric molecule. Taken together, these data suggest that Shc, by interacting with the phosphorylated ζ chain on the one hand and with Grb2 and mSOS on the other, may couple T cell activation to the Ras signaling pathway.

I 252 Association of p56^{lck} with Casein Kinase II through its *src* - Homology 2 Domain. Stéphane Richard, David W. Litchfield*, Patricia A. Connelly**, and Andrey S. Shaw. Department of Pathology, Washington University School of Medicine, St. Louis, MO, 63110. *Manitoba Institute of Cell Biology, Winnipeg, MB, R3E 0V9.**Pfizer Inc., Central Research Division, Groton, CT, 06340.

All src-like tyrosine kinases, including p56^{lck}, contain tandemly linked src-homology 3 (SH3) and src-homology 2 (SH2) domains. These non-catalytic regions are thought to mediate protein-protein interactions. We, therefore, sought to identify specific p56^{lck} SH3 binding proteins by using a fusion protein containing the SH3 and SH2 domains of p56^{lck}. The p56^{lck} fusion protein bound a set of cellular proteins which were not bound by a similar fusion protein containing a mutation in its SH3 domain. One of the proteins had a kinase activity with the characteristics of casein kinase II (CKII). This interaction was direct since the p56^{lck} fusion protein could bind purified CKII. Mapping studies demonstrated that the SH2 domain of p56^{lck} was sufficient to bind to the beta subunit of CKII with high affinity. This was specific because mutation or deletion of critical residues in the SH2 domain of the p56^{lck} fusion protein abolished CKII binding. Interestingly, changing critical residues in the SH3 domain also reduced the binding of CKII to the p56^{lck} fusion protein. Our results suggest that SH2 domains may link src-family tyrosine kinases to serine-threonine kinases and that these interactions may be regulated by the SH3 domain.

I 253 IDENTIFICATION AND FUNCTION OF THE PHOSPHORYLATION OF THE C-TERMINUS OF FOCAL ADHESION KINASE, Alan Richardson, Michael. D. Schaller, Jeffrey D. Hildebrand, J. Thomas Parsons, University of Virginia, Dept. of Microbiology, Charlottesville Va 22908.

Focal adhesion kinase (FAK) is a cytoplasmic tyrosine kinase that was first identified by virtue of its increased tyrosine phosphorylation in cells transformed by activated variants of the protein kinase Src. Recent work has shown that it is recruited to focal adhesions in response to the clustering of integrins that is brought about by cell adhesion to the extracellular matrix. It is likely that FAK plays a pivotal role both in linking the extracellular matrix to the cytoskeleton and in signal transduction by integrins. In chicken embryo fibroblasts, the C-terminal non-catalytic domain of FAK, termed FRNK, is expressed separately. While activation of integrins causes tyrosine phosphorylation of FAK in its N-terminal region, FRNK is also phosphorylated and undergoes a mobility shift from 41kD to 43 kD. The identity of FRNK with the C-terminal domain of FAK suggests that their phosphorylation will be co-ordinately regulated.

We are investigating the role of these phosphorylation events in regulating the function of FAK and FRNK. We will present data from metabolic labeling studies that FRNK is phosphorylated in both its 41kD and 43kD forms. A higher molecular weight form, p44, was also identified and its homology with p41 and p43 shown by proteolytic mapping. Phosphoamino acid analysis indicated that phosphorylation of FRNK occurs primarily on serine. Lysates prepared from cells adherent to fibronectin can also phosphorylate FRNK. We have identified a kinase that reproduces these phosphorylation events *in vitro* and have used this to identify the site(s) of phosphorylation. Current studies are aimed at elucidating their role.

I 254 THE C. ELEGANS daf-7 GENE ENCODES A NEW MEMBER OF THE TRANSFORMING GROWTH FACTOR-ß SUPERFAMILY THAT IS A PUTATIVE LIGAND FOR THE daf-1 RECEPTOR, Donald L. Riddle, Chang-Su Lim, Robert Johnsen, Miguel Estevez, Patrice S. Albert, and David Pilgrim*, Division of Biological Sciences, University of Missouri, Columbia, MO 65211; *Dept. of Genetics, University of Alberta, Edmonton, Alberta, Canada T6G 2E9

The C. elegans dauer larva is a developmentally arrested, non-feeding dispersal stage that is formed in response to overcrowding and limited food. Mutations in genes daf-1, daf-4 and daf-7 result in constitutive formation of dauer larvae in abundant food. These genes have been cloned by transposon tagging. The daf-1 receptor ser/thr kinase was the first receptor of its type to be identified (Georgi et al., Cell 61:635-645, 1990). The activin and TGF-ß type II receptors were subsequently found to be similar to the daf-1 receptor in their kinase domains and in a cysteine-rich extracellular motif. The daf-4 receptor is yet another member of this receptor family, and it has been shown to bind human bone morphogenetic protein (BMP)-2 and BMP-4 when expressed in monkey COS cells (Estevez et al., Nature 365, in press). It is the first receptor identified for the BMP group of ligands. The daf-1 receptor on the other hand, does not bind BMPs, activin or TGF-ß.

The deduced daf-7 gene product is a candidate for the daf-1 ligand. Both cDNA and genomic sequences have been determined. The encoded protein includes a hydrophobic leader sequence, protease cleavage site, and the seven cysteines strictly conserved in the TGF-ß superfamily. The daf-7 protein is most similar to human BMP-4 and Drosophila decapentaplegic. However, the mature protein is only about 30% identical to them, and it lacks several amino acids conserved in this group. We conclude that daf-7 encodes a novel member of the TGF-ß superfamily. These 110- to 120-amino-acid growth factors control a wide array of cellular processes in vertebrates, including cell proliferation, differentiation and organization. Some of the daf-7 mutations are missense amino acid substitutions that affect highly conserved residues, and one mutation is a 300 bp deletion removing the protease cleavage site and five conserved cysteines. A developmental northern blot showed that the daf-1 and daf-4 mRNAs are expressed in all developmental stages, whereas daf-7 is expressed in L1 and L2 larvae, when the commitment to non-dauer development is made. Transcriptional regulation of the daf-7 gene in chemosensory neurons may regulate dauer vs. non-dauer development, which is partially mediated by the C. elegans analogue of a BMP signalling system.

I 255 MET-HGF/SF AUTOCRINE STIMULATION AND SARCOMA TUMORIGENICITY, Sing Rong, Michael Jeffers, James H. Resau, Ilan Tsarfaty, Marianne Oskarsson, and George F. Vande Woude, ABL-Basic Research Program, P.O. Box B, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702

The ligand for the *met* protooncogene product (Met) receptor tyrosine kinase is hepatocyte growth factor/scatter factor (HGF/SF). HGF/SF and Met constitute a paracrine signalling system in which cells of mesenchymal origin produce the ligand, which binds the receptor predominantly expressed in cells of epithelial origin. We have shown that mouse NIH/3T3 fibroblasts over-expressing the *met* protooncogene induce tumor formation in nude mice via an autocrine mechanism (Rong *et al.*, Mol. Cell Biol., 12:5152-5158, 1992). The highly tumorigenic behavior of these cells led us to examine human cell lines established from various sarcomas for Met expression. Low-passage, non-immortalized human primary fibroblast cultures and a variety of human sarcoma cell lines were analyzed for Met and HGF/SF expression. We observed that Met expression was much higher in the sarcoma cell lines than in primary fibroblast cultures. Moreover, in most of the sarcoma cell lines, Met was readily detected with an anti-phospho-tyrosine antibody (Anti-P-Tyr). HGF/SF was detected in several of the tumor cell lines but the level was lower than in primary fibroblasts. Addition of exogenous HGF/SF to serum-starved cells increases the ^{3}H-thymidine uptake for several human sarcoma cell lines, which supports the idea that Met-HGF/SF signalling may play a role in the tumorigenicity of these cells by facilitating their growth. Based on the novel finding that Met is expressed at low levels in fibroblasts, we propose that Met-HGF/SF autocrine stimulation may be a fundamental property of non-immortalized fibroblast cells in culture. Furthermore, our studies suggest that Met overexpression in cells of mesenchymal origin may be an important oncogenic mechanism in certain soft tissue sarcomas.

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I 256 CONSERVED STRUCTURAL DOMAIN "TREE ANALYSIS" OF THE PROTEIN KINASE DATABASE: APPLICATION TO THE PREDICTION OF RELATIVE SUBSTRATE SPECIFICITY FOR NOVEL RECEPTOR KINASES Jan S. Rosenbaum and Paul E. Correa, The Procter & Gamble Company, Corporate Research Division, Miami Valley Laboratories, P.O. Box 398707, Cincinnati, OH 45239-8707

When faced with a protein sequence encoding a protein of unknown function, it is often useful to compare predicted structural elements to those present in proteins of known function. In order to do this for proteins that are predicted to encode novel receptor protein kinases, we have developed a computer program package that pre-aligns the entire protein kinase database according to a consensus kinase domain pattern. This pattern is derived from an alignment of known kinases from a variety of species, and stresses conserved residues in kinase domains I through IX which appear to serve discrete functions in the enzymatic mechanism; as discerned from the crystal structure of the catalytic subunit of cAMP dependent protein kinase (cAPK). The entire kinase database is then grouped according to this pattern using a cutoff of 85% similarity. Cross-comparisons between the different subgroups allows us to discern the relationships between the different kinase domain sequences. As expected, we have found that the relative similarity observed in the cross-comparisons changes as the analysis proceeds from kinase domains I-IX to include kinase domains X, XI, and the carboxy terminus. Since kinase domains X, XI, and the carboxy terminus "wrap around" the catalytic core of the kinase in the cAPK structure we have interpreted these results to mean that kinase domains X, XI, and the carboxy terminus contain amino acid residues that will influence substrate accessibility to the catalytic core. For example, the insulin receptor bears a high degree of relative similarity to both ser/thr and tyrosine kinases when only kinase domains I-IX are analyzed, but extension of the analysis to include the carboxy terminus results in similarity to only the tyrosine kinases and predominantly to the receptor tyrosine kinases. Analysis of MAPKK, a known dual-specificity kinase, reveals an increased relative similarity to dual-specificity kinases as the analysis proceeds throughout the carboxy terminus. Application of this analysis to the TGF-β, activin, DAF-1, DAF-4, and the "Type I" TGF-β, activin, and orphan receptors reveals a dual-specificity character in the kinase domain not recognized by conventional analysis programs. Interestingly, the DAF-1 and "Type I" receptors possess an increased relative similarity to the receptor and cytosolic tyrosine kinases in comparison to the "Type II" receptors for TGF-β and activin.

I 257 NEUROTROPHIN RECEPTOR EXPRESSION AND RESPONSIVENESS IN DEVELOPMENT AND MAINTENANCE OF THE RAT OLFACTORY SYSTEM. A. Jane I. Roskams, M. Angelyn Bethel and Gabriele V. Ronnett. Departments of Neuroscience and Neurology, Johns Hopkins School of Medicine, 725 N. Wolfe Street, Baltimore MD 21205.

The neurotrophins, nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and the neurotrophins - 3,4 and 5 (NT-3,4,5) have been identified and characterized by their ability to support neuronal survival. The interaction of these factors with the Trk family of receptor tyrosine kinases is complex and involves them acting either individually through one Trk, through a complex of Trks or through a Trk complexed with the low affinity NGF receptor (LNGFR). The neurotrophins and their receptors are expressed widely in the developing and maturing central nervous system (CNS). *In vitro* studies have indicated that neuronal cells are differentially responsive to the neurotrophins at different stages of differentiation. The olfactory neuronal epithelium is unique in its ability to undergo neuronal regeneration throughout the lifetime of the organism. As such, neurons at different stages of maturation are found throughout the olfactory epithelium in the adult rat. In addition, primary cultures of olfactory neurons appear to be functionally dependent on a ready supply of NGF. Using primers directed against unique sequences of the LNGFR, Trk, Trk B and Trk C, we performed a nested modification of the reverse transcription - polymerase chain reaction (RT-PCR) to identify transcripts of each of these receptors in RNA prepared from olfactory epithelial tissue and primary cultures of olfactory neurons. The identity of rat Trk A, B and C was confirmed by sequencing of the subcloned PCR products and western blotting. Using a double bulbectomy paradigm designed to monitor olfactory neuron cell death and regeneration, we have examined how expression of each of the neurotrophin receptors changes at the protein, RNA and immunohistochemical level as a result of this treatment.

I 258 PHOSPHATIDYLINOSITOL 3-KINASE AND 4-KINASE BIND TO THE CD4-p56^{lck} COMPLEX: SH3 DOMAIN BINDING TO PI 3-KINASE, BUT NOT PI 4-KINASE. Christopher E. Rudd, Rosana Kapeller, Ottmar Janssen, Lewis. C. Cantley K. V. S. Prasad Division of Tumor Immunology, Dana-Farber Cancer Institute, Boston, 02115 and Departments of Pathology and Cellular and Molecular Physiology, Harvard Medical School, Boston. Division of Signal Transduction, Beth Israel Hospital, Boston

CD4 serves as a receptor for MHC class II, the Human Immunodeficiency Virus (HIV-1) and is coupled to the protein-tyrosine kinase p56^{lck}. In addition to the protein-tyrosine kinase domain, p56^{lck} possesses Src-homology 1 and 2 [SH2 and SH3] domains as well as an unique N-terminal region. The mechanism by which p56^{lck} generates intracellular signals is unclear, although it has the potential to interaction with various downstream molecules. One such downstream target is the lipid kinase phosphatidylinositol 3-kinase (PI 3-kinase), which has been found to bind to activated pp60^{src} and receptor-tyrosine kinases. Both PI -3 kinase and PI 4-kinase was found to associate with the CD4:p56^{lck} complex as judged by the presence of PI 3-P and PI 4-P in anti-CD4 immunoprecipitates and detected by high pressure liquid chromatographic (HPLC) analysis. The level of associated PI 4-kinase was generally higher than PI 3 kinase activity. HIV-1 gp120 and antibody-mediated crosslinking induced a 10-15 fold increase in level of CD4 associated PI 4- and PI 3-kinase. The use of Glutathione S-transferase (GST) fusion proteins carrying *lck*-SH2, *lck*-SH3 and *lck*-SH2/SH3 domains showed PI 3 kinase binding to the SH3 domain of p56^{lck}, an interaction facilitated by the presence of an adjacent SH2 domain. The SH3 domain bound directly to the p85 subunit. PI 4-kinase bound to neither the SH2 nor the SH3 domain of p56^{lck}. CD4-p56^{lck} contributes PI 3-and PI 4-kinases to the activation process of T cells and may play a role in HIV-1 induced immune defects.

I 259 INVOLVEMENT OF CSK AND SRC IN THE CELL-TO-SUBSTRATUM ADHESION DEPENDENT PROTEIN TYROSINE PHOSPHORYLATION, Hisataka Sabe and Hidesaburo Hanafusa, Laboratory of Molecular Oncology, The Rockefeller University, New York, NY 10021-6399

Increasing evidence suggests involvement of tyrosine phosphorylation of intracellular proteins during cell adhesion to extracellular matrix (ECM), which might lead to the regulation of cell growth, diferentiation and transformation. The first evidence has come from the finding that the p120 protein, whose tyrosine phosphorylation occurs in a cell adhesion-dependent manner, is Focal Adhesion Kinase, FAK. Another focal adhesion protein, paxillin, was then shown to be tyrosine phosphorylated in a similar way. Since Csk (C-terminal Src Kinase), which has been suggested as a negative regulator of Src-family tyrosine kinases, binds to the tyrosine-phosphorylated forms of FAK and paxillin via its SH2 domain, we have suggested that Csk might have some role in cell adhesion, including the suppression of the kinase activity of a certain fraction of c-Src which might function at the focal adhesion plaques. We here show that Csk phosphorylates paxillin at an early stage of cell adhesion to ECM and binds to this phosphorylated form. Suppression of Csk causes an activation of Src-family kinases, and this activation leads to phosphorylate paxillin with quite different isoforms from that induced by Csk. Thus, the suppression of Src kinases by Csk changes the phosphorylation of paxillin from Src-type to Csk-type, while preventing cell transformation. We will discuss the interaction of Csk, c-Src, Fak and paxillin concerning the regulation of the architecture of the adhesion plaques. Moreover, we report that a series of focal adhesion proteins, in addition to Fak and paxillin, are also tyrosine-phosphorylated in a cell adhesion dependent manner, in normal and Src-transformed fibroblasts. Cellular tyrosine phosphorylation of transformed cells growing in anchorage-independent manner was also analyzed. The physiological significance of these cell adhesion dependent tyrosine phosphorylations and their relation to the oncogenic/anchorage-independent growth will also be discussed.

I 260 EVIDENCE FOR A FUNCTIONAL ROLE OF SHC PROTEINS IN MITOGENIC SIGNALING INDUCED BY INSULIN, INSULIN LIKE GROWTH FACTOR-1, AND EPIDERMAL GROWTH FACTOR, Toshiyasu Sasaoka, David R. Rose, Byung H. Jhun and Jerrold M. Olefsky, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, CA 92093 and the VA Medical Center, San Diego, CA 92161

Shc proteins contain a single SH2 domain, lack catalytic activity, and are substrates for activated receptors for insulin, IGF-1, and EGF. Treatment with these growth factors induces rapid tyrosine phosphorylation of Shc proteins. To investigate the potential role of Shc in mitogenic signaling, we microinjected affinity purified anti-Shc antibodies into living Rat1 fibroblasts overexpressing human insulin receptors. BrdU incorporation into newly synthesized DNA was subsequently studied to assess the importance of Shc in cell cycle progression. The anti-Shc antibody specifically recognized the 46, 52, and 66 kDa isoforms of the Shc protein and immunoprecipitated more than 90% of total cellular Shc. Cellular microinjection of the anti-Shc antibody inhibited the stimulatory effect of insulin, IGF-1, or EGF by 81%, 70%, or 81%, respectively. In contrast, the mitogenic effect of fetal calf serum was not inhibited by microinjection of the anti-Shc antibody. Microinjection of an oncogenic p21ras protein into quiescent cells produced constitutively active mitogenic signaling, leading to maximal rates of BrdU incorporation. Co-microinjection of the oncogenic mutant p21ras protein with the anti-Shc antibody, restored insulin and EGF stimulated DNA synthesis to normal. These results indicate that Shc proteins are an important component, and lie downstream of tyrosine kinase receptors but upstream of p21ras, in a mitogenic signal transduction pathway that is shared by insulin, IGF-1, and EGF.

I 261 **CHARACTERIZATION OF CELLULAR PROTEINS ASSOCIATED WITH TEC SH3 DOMAIN IN A HEMATOPOIETIC CELL LINE,** Ken Sato[1,2], Hiroyuki Mano[1,2], Yoshio Yazaki[2],, and Hisamaru Hirai[1,2], [1]Department of Molecular Biology, Jichi Medical School, Tochigi, 329-04 JAPAN, [2]Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, JAPAN

The cytoplasmic protein-tyrosine kinase, Tec, is widely expressed in mouse hematopoietic cell lines. Its kinase activity and phosphorylation level increases by the stimulation of IL-3 in an IL-3-dependent cell line, FDC-P1. These facts suggest that the Tec kinase might be involved in IL-3 signaling pathway. SH3 domain is presumed to play a pivotal role in mitogenic signaling pathway. Interestingly, there are two different forms of the Tec SH3 domain generated by alternative splicing. One (SH3L) has five ß strands comparable to known SH3 domains. On the other hand, another Tec SH3 domain (SH3S) lacks C-terminal two ß strands completely. To investigate the differential function of these two SH3 domains in intracellular signal transduction pathway, we analysed cellular proteins associated with SH3L or SH3S, using the GST-fusion system. From the total cell lysates of FDC-P1 metabolically labeled with [^{35}S]methionine, two cellular proteins of approximately 58-KDa and 54-KDa were bound only to GST-SH3L, but not to GST-SH3S or GST. In the case of [^{32}P]orthophosphate labeling, phosphoprotein(s) of approximately of 58-KDa were associated only with GST-SH3L. These association was not affected by IL-3 stimulation. Characterization of these proteins and the implication of these differential binding is currently under investigation.

I 262 **HERBIMYCIN A INHIBITS THE IGF-I RECEPTOR PROTEIN TYROSINE KINASE AND CELLULAR PROLIFERATION IN HUMAN BREAST CANCER CELLS,** Laura Sepp-Lorenzino, Zheng-ping Ma, Neal Rosen, and David E. Lebwohl, Department of Medicine and the Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

Insulin and insulin-like growth factors (IGFs) are potent mitogens for many breast cancer cell lines and may play an important role in the pathogenesis of human breast cancer. Previous work suggests that IGF-II is expressed in stromal fibroblasts derived from breast tumors and may regulate the growth of malignant breast cells in a paracrine manner. In cell lines which are responsive to IGFs, including MCF-7, the proliferative signal is transduced by the IGF-I receptor (IGFIR). The tyrosine kinase of the IGFIR represents a potential target for new therapeutic agents. Thus, we are testing tyrosine kinase inhibitors and studying their effects on signalling via this and other receptors.

Herbimycin A is a benzenoid ansamycin antibiotic which reverses morphological transformation by cytoplasmic tyrosine kinase oncogenes, such as v-*src*, with an accompanying decrease in the kinase activity of the oncoproteins. In the MCF-7 line, stimulation with insulin (µM) or IGFs (nM) resulted in rapid tyrosine phosphorylation of the ß subunit of the IGFIR, its 185 kDa substrates, and the 120 kDa p21^{ras}-GTPase activating protein (GAP). Pretreatment of cells with herbimycin A for 24 hr effectively diminished IGFIR autophosphorylation as well as pp185 and GAP tyrosine phosphorylation in a parallel dose-dependent fashion (0.1 to 10 µg/ml). These events correlated with an inhibition of cellular proliferation in the presence of fetal bovine serum, with complete inhibition at 1 µg/ml. The mechanism of kinase loss was studied in these cells. Herbimycin A had no effect on immunopurified IGFIR kinase in an *in vitro* kinase reaction under standard conditions (including reducing agents.) *In vivo*, however, the quantity of receptor kinase protein was markedly diminished within 4 hr of herbimycin A treatment. Similar effects on receptor protein content was observed in cells expressing EGF and insulin receptor, but minimal effect was observed on the quantity of other proteins including p85-PI-3 Kinase. We conclude that herbimycin A treatment of breast cancer cells causes a loss of receptor protein and kinase activity, which may lead to the inhibition of cellular proliferation mediated by receptor tyrosine kinases.

I 263 THE ISOLATION AND ANALYSIS OF α2 - CHIMAERIN, AN SH2-CONTAINING GTPASE ACTIVATING PROTEIN FOR RAC. Wun Chey Sin[1,2], Christine Hall[1] & Louis Lim[1,2]. [1]Department of Neurochemistry, Institute of Neurology, 1 Wakefield Street, London WC1N 1PJ; [2]Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511.

n(α1)-Chimaerin is a p21^{rac} GTPase activating protein(GAP) and a phorbol ester receptor that is specifically expressed in the brain. We have now isolated a variant cDNA (designated α2) encoding an SH2 domain at its N-terminal in addition to the phorbol ester binding and Rac-GAP catalytic domain. The full length α2-chimaerin mRNA sequence (2.1 kb) was obtained from overlapping cDNAs and by 5'RACE PCR of human brain cDNA. Various 5'RACE products obtained showed sequence heterogeneity, possibly indicating further alternate exon usage.

SH2 domains bind selective phosphotyrosine containing proteins. Ras-GAP, for example interacts with activated PDGF and EGF receptor via its SH2 domain. In contrast, bacterial expressed GST-α2-SH2 fusion protein does not bind activated EGF receptor. However, the SH2 fusion protein was able to bind several phosphoproteins (120, 80, 60, 38 kDa) in PC12 cells. Phosphorylation of these proteins was increased when PC12 cells were treated with growth factors. The characteristics of these binding proteins are currently being investigated. With an SH2 domain at its N-terminal, α2-chimaerin is anticipated to interact with regulatory cellular components distinct from those of α1 in the signalling pathway.

References: Hall,C. *et al* (1993) Mol. Cell. Biol. **13**:4986-4998; Hall,C. *et al* (1990) J. Mol. Biol. **211**,11-16; Ahmed,S *et al* (1990) Biochem. J. **272**, 767-773; Diekmann,D. *et al* (1991) Nature **351**, 400-402

I 264 LOCATION OF CYCLIC AMP-DEPENDENT PROTEIN KINASE TYPE I WITH THE TCR/CD3 COMPLEX

Bjørn S. Skålhegg[1,2], Kjetil Taskén[2], Henrik S. Huitfeldt[3,4], Tor Lea[1], Tore Jahnsen[2], and Vidar Hansson[2]

1) Institute of Immunology and Rheumatology, Rikshospitalet, Oslo and 2) Institute of Medical Biochemistry, University of Oslo, Oslo, 3) National Institute of Public Health, Oslo, 4) LIIPAT, Institute of Pathology, Rikshospitalet, Oslo, Norway.

The recent demonstration of multiple regulatory (R) subunits of cAMP-dependent protein kinase (cAK) showing cell specific expression and regulation as well as distinct intracellular compartmentalization, gave support for the idea that different functions of cAMP may be mediated via specific isozymes of cAK. We demonstrate that cAMP-dependent inhibition of human T-cell proliferation induced through the antigen-specific T-cell receptor CD3 (TCR/CD3) complex is specifically mediated via the cytosol associated cAKI ($RI\alpha_2C_2$), but not the particulate associated cAKII ($RII\alpha_2C_2$). Furthermore, we demonstrate both by immunocytochemistry and immunoprecipitation that cAKI but not cAKII localizes to and interacts with the TCR/CD3 complex during T-cell activation and capping. We suggest that the cAKI ($RI\alpha_2C_2$) localized to the TCR/CD3 complex, upon stimulation by cAMP releases kinase activity which through phosphorylation causes uncoupling of the TCR/CD3 complex from the intracellular signalling systems.

I 265 BTK, THE PROTEIN-TYROSINE KINASE MUTATED IN X-LINKED AGAMMAGLOBULINEMIA

C. I. Edvard Smith, Igor Vorechovsky, David Vetrie, Berivan Baskin, Lennart Hammarström, Lars Brandén, Susanne Müller, Hina S. Maniar, Khalid B. Islam, and Paschalis Sideras.
Center for BioTechnology, Karolinska Institute, NOVUM, S-14157 Sweden and Division of Medical and Molecular Genetics, Guy's Hospital, Guy's Tower, London SE1 9RT, UK.

The human primary immunodeficiency, X-linked agammaglobulinemia (XLA), is characterized by a B-lymphocyte differentiation defect causing agammaglobulinemia and increased susceptibility to infections in affected males. We have recently used a positional cloning strategy, based on cDNA enrichment on yeast artificial chromosomes, for the isolation of this gene (*Nature* 361:226, 1993) and in a collaborative study demonstrated that the same gene is mutated in the mouse strain *xid* (*Science* 261:355, 1993). The gene was found to encode a novel protein-tyrosine kinase and was named *Btk* (formerly *Atk* or *Bpk*).
The kinase is related to the Src family of kinases, having a single SH1, SH2 and SH3 domain, but differs in that it lacks a myristylation site as well as a carboxyl-terminal regulatory tyrosine residue, and has a considerably longer 'unique' domain. In order to further characterize XLA we have investigated the expression pattern of the *Btk* gene at the level of mRNA and protein. The gene is expressed in most hematopoietic cells, including mast cells and progenitor cells expressing the early differentiation marker CD34, and is specifically down-regulated in T lymphocytes and plasma cells. We are presently carrying out several studies, including mutation analysis and functional characterization of the protein and these data will be presented.

I 266 THE *ltk* TRANSMEMBRANE TYROSINE KINASE MAY BE PART OF AN OLIGOMERIC RECEPTOR,

Allard J. Snijders and André Bernards, Molecular Genetics Laboratory, Massachusetts General Hospital Cancer Center and Harvard Medical School, Building 149, 13th street, Boston, MA 02129

Ltk is a transmembrane (TM) tyrosine kinase whose kinase domain is related to that of *ros* and *sevenless*. Four tissue-specific *ltk* mRNAs have been identified. These mRNAs arise through alternative splicing and alternative promoter usage and predict proteins that differ upstream of the TM segment. One of the *ltk* proteins has been reported to reside in the endoplasmic reticulum (ER), where its kinase activity may respond to changes in cellular redox potential. To analyse why *ltk* resides in the ER, we generated and transiently expressed *ltk* mutants in COS cells. Whereas wildtype *ltk* remained in the ER, a hybrid protein in which the first 20 amino acids downstream of the TM segment were replaced with the corresponding segment of the EGF receptor was surface expressed. The juxtamembrane domain of *ltk* thus either contains an ER retention signal or is required for the correct folding and assembly of an oligomeric receptor. The latter may be the case for two reasons. First, in *ltk* transgenic mice that over-express one form of *ltk* protein up to 1000-fold, the protein is expressed on the surface of thymocytes. Secondly, we have found that only about 50% of *ltk* protein in transfected COS cells has the expected orientation with the kinase domain in the cytoplasm, whereas the other 50% has the type 2 orientation with the kinase domain in the lumen of the ER. This indicates that *ltk* does not contain the information required to insert itself correctly into the membrane, suggesting a second subunit may be required to attain this.

I 267 ONCOSTATIN M ACTIVATES PHOSPHATIDYL - INOSITOL-3-KINASE IN KAPOSI'S SARCOMA CELLS.

Raffaella Soldi*, Andrea Graziani+, Roberto Benelli §, Amalia Bosia* Adriana Albini§ ,F.Bussolino*
*Dipartimento di Genetica, Biologia e Chimica Medica and +Dipartimento di Scienze Biomediche e Oncologia, Torino. §Istituto Nazionale per la Ricerca sul Cancro, Genova, Italy.

Oncostatin M (OM) is a polypetide cytokine that induces autocrine and paracrine effects on AIDS-Kaposi's sarcoma (KS) cells (Nair et al., Science, 255:1430-1432, 1992; Miles et al., Science, 255:1432-1434, 1992). The signaling pathways underlying this activation are unknown. We have found that nanomolar concentrations of OM induced a rapid tyrosine phosphorylation of multiple proteins in KS cell, including the p85 regulatory subunit of phosphatidyl- inositol-3-kinase (PI3K) and of a 173 KDa protein. In addition, immune complex kinase assay performed with anti-cst.1 antibody , which recognises $pp60^{src}$, $pp62^{yes}$, and $pp59^{fyn}$, showed the activation of a tyrosine kinase activity. These effects of OM on KS cells were accompanied by the activation of PI3K and the rapid synthesis of PI 3,4-biphosphate. PI3K activity in antiphosphotyrosine immunoprecipitates increased 10-to-30 fold after OM stimulation (10 min.). Interestingly a small but significant amount of the enzyme activity is associated with *src* kinase family. These data suggest that OM utilises tyrosine phosphorylation signal transduction pathway in KS cells involving the activation of PI3K.

I 268 SUBSTRATE SPECIFICITY OF PROTEIN KINASES
Zhou Songyang, Helen Piwnica-Worms, and Lewis C. Cantley, Cellular and Molecular Physiology, and Department of Microbiology, Harvard Medical School, Boston, MA02115
Protein phosphorylation by protein kinases is one of the most important process in cell proliferation. Individual kinases recognize a unique set of substrates leading to specific cellular responses. To understand how protein kinases select their cellular targets, we developed a novel technique to quickly predict the substrate specificity using degenerate peptide libraries. Peptides degenerate at four amino acids upstream and downstream of the tyrosine or serine were phosphorylated by kinases. Phosphorylated peptides were separated, sequenced and compared to the original mixture to determine the specificity. Our predicted optimal motifs for serine kinases include cAMP dependent kinase, PKC, and CDC2 are in gree with previous findings. The specificity of tyrosine kinases (v-src, lck, and EGF receptor) were also determined, demonstrating that these kinases have distinct substrate specificity. In general, residues at one and three amino acids C- or N-terminal to the tyrosine are critical for good substrates for tyrosine kinases. The identification of optimal peptide substrates for kinases provides a tremendous short-cut in finding physiological targets of kinases as well as suitable peptide substrates and inhibitors for study of these enzymes.

I 269 SIGNAL TRANSDUCTION BY THE TRK/NERVE GROWTH FACTOR RECEPTOR.
Robert M. Stephens[1], David M. Loeb[2], Terry D. Copeland[1], Lloyd R. Greene[2] and David R. Kaplan[1]. [1]ABL-Basic Research Program, NCI-FCRDC, Frederick, MD 21702, [2]Dept. Pathology, Columbia University, N.Y., N.Y., 10032.
The Trk family of receptor tyrosine kinases mediate the develoment and survival of neurons. To examine the mechanism of neuronal signal transduction used by Trk, we identified three proteins, SHC, PLC-γ1, and PI-3 kinase that form complexes with ligand-activated Trk in PC12 cells and neurons. To determine the role of the Trk substrates in NGF responses, we identified the sites of Trk tyrosine autophosphorylation and substrate binding. These sites were mutated, and the mutant Trk receptors expressed in PC12-derived cells that lack endogenous Trk (nnr5). Single unique sites were identified for SHC and PLC-γ1. The interactions of SHC and PLC-γ1 with Trk were specific and rapid, and required tyrosine phosphorylation at the Trk interaction sites.

Expression of SHC or PLC-γ1 Trk interaction mutants in nnr5 cells resulted in normal neurite outgrowth responses to NGF, indicating that either of the interaction sites is dispensible for receptor function. However, a double mutant that lacked both of these sites was partially defective in the initiation of NGF-induced differentiation and failed to maintain the differentiated phenotype. While single interaction site mutants were capable of inducing the tyrosine phosphorylation and activation of MAPK in response to NGF, the double interaction site mutants were defective in eliciting these events. We postulate that Trk receptors can activate MAPK by either an SHC or PLC-γ1-dependent signalling pathway. Elimination of both of these pathways inhibits MAPK activation and the appearance of stable neurites. These results suggest a model whereby Trk receptors utilize redundant signal transduction pathways to mediate NGF responses.

In order to identify other sequences in Trk that transmit NGF signals, we constructed Trk mutants that lacked residues conserved among all Trk family members. One class of mutants showed delayed neurite outgrowth responses, while a second class failed to exhibit NGF-induced differentiation but was competent in mediating NGF-induced neuronal survival. These mutants will allow us to characterize the signalling molecules responsible for different aspects of neuronal development.

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I 270 **Alteration of p56^{lck} SH2 Domain Peptide Binding Specificity by the Unique N-Terminal Region**
Lesley Stolz, Gillian Payne, David Winkler, Jaekyoon Shin*, Christopher Walsh, Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, *Department of Tumor Virology, Dana-Farber Cancer Institute, Boston, MA 02115

The Src family of tyrosine kinases have a non-conserved N-terminal region followed by the conserved Src homology 3 (SH3) and Src homology 2 (SH2) domains. The SH3 and SH2 domains are thought to bind effector proteins, recognizing proline rich and tyrosine phosphorylated regions of polypeptides respectively. When the p56^{lck} SH2 domain is expressed with the SH3 domain and a portion of the non-conserved N-terminal region including a serine phosphorylation site (S59), we find altered phosphotyrosine peptide binding specificity. However the specificity of a construct containing just the SH2 and SH3 sequences resembles that of the isolated SH2 domain. Similar constructs have been made for Fyn and Src members of this family. These proteins show different behavior from Lck indicating a potential difference in structure-function relationships within the Src family of kinases. These differences have been measured directly and by competition assay using an in vitro surface plasmon resonance assay. This finding indicates that the functions of the p56^{lck} Src homology regions are modified in the context of the unique N-terminal region and suggests a mechanism for regulation of p56^{lck} activity by N-terminal serine phosphorylation.

I 271 COLLAGEN INDUCED COLLAGENASE PRODUCTION: SECOND MESSENGERS AND PROMOTER RESPONSIVE ELEMENTS,
Barry D. Sudbeck, Ulpu K. Saarialho-Kere, Howard G. Welgus, William C. Parks and Alice P. Pentland, Division of Dermatology, Jewish Hospital and Washington University, St. Louis, St. Louis, MO 63110

Matrix metalloproteinases constitute a family of endopeptidases which can degrade nearly all components of the extracellular matrix. One member, interstitial collagenase, possesses the unique ability to cleave native fibrillar type I collagen, the most abundant matrix protein of the dermis. We have shown that collagenase is expressed predominantly by basal keratinocytes actively migrating over the dermal matrix in chronic and acute wounds, but the enzyme is not produced by epidermal cells in contact with an intact basement membrane. To begin understanding the mechanism of this matrix-influenced induction, we are studying collagenase regulation in cultured keratinocytes. Reflecting our *in vivo* observations, collagenase production is upregulated in keratinocytes grown on type I collagen, but only basal levels are detected in keratinocytes grown on basement membrane proteins (Matrigel) or denatured collagen (gelatin). These findings suggest that the inductive effect of collagen is mediated by the $\alpha_2\beta_1$ integrin receptor which recognizes native and denatured type I collagen and is constitutively expressed by epidermal cells. Consistent with an integrin-mediated induction, collagenase production was blocked by tyrosine kinase inhibitors. Induction of collagenase expression by phorbol ester (PMA), which stimulates collagenase production independent of the matrix substratum, was partially inhibited by the tyrosine kinase inhibitor genistein. These findings indicate that tyrosine kinases are involved in collagenase expression in keratinocytes grown on collagen or stimulated by PMA. Collagenase promoter studies indicate an element(s) within 2 kB of the initiation site are responsive to collagen. Currently, we are examining the role of specific integrins and intracellular kinases to determine their role in the matrix induction of the collagenase gene and delineating further the collagen-responsive elements within the collagenase promoter.

I 272 SRC SIGNALLING IN MITOSIS: INTERACTIONS BETWEEN A 68 kD SUBSTRATE AND THE SRC SH2 AND SH3 DOMAINS, Stephen J. Taylor and David Shalloway, Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, NY 14853.

c-Src activity is repressed in vivo by intra- and possibly inter- molecular protein-protein interactions. Under certain physiological circumstances, c-Src activation is elicited by disruption of these interactions, by dephosphorylation of its regulatory phosphotyrosine residue and/or by interaction with SH2 or SH3 domain binding proteins. In the case of fibroblast mitosis, c-Src activation entails both an increase in tyrosine kinase activity and an enhanced ability to bind phosphotyrosine-containing ligands via its SH2 domain, as measured in vitro. Since the relevant targets of physiologically activated c-Src have not been identified we have investigated potential substrates/binding partners of c-Src in mitosis. Increased tyrosine phosphorylation of a 68 kD protein(s) (p68) was observed in fibroblasts overexpressing normal or mutationally-activated c-Src when arrested in mitosis. Tyrosine phosphorylated p68 formed a stable complex with Src, as determined by co-immunoprecipitation from mitotic cell lysates. c-Src mutants bearing deletions within the SH2 or SH3 domains failed to fonn a stable complex with p68 and co-immunoprecipitation of p68 with Src was blocked in the presence of a recombinant fusion protein containing the SH3 and SH2 domains of c-Src, but not one containing SH3 alone. However, p68 (identified by its mitotic phosphorylation) bound to the GST-SH3 fusion protein in lysates and directly on Western blots in a phosphotyrosine independent manner. From immunoprecipitation/immunoblotting analysis using specific antibodies we have been unable to identify p68 as a number of candidate proteins, including paxillin, SHPTP2, Raf-1, and $p66^{shc}$.

The results suggest that a Src-p68 complex may play a signalling role in mitosis and perhaps under other physiological conditions. Furthermore, the SH3 binding properties of p68, and the apparent involvement of SH3 in p68 tyrosine phosphorylation and SH2 binding, suggest that the SH3 domain may play an effector role in c-Src function, in addition to its negative regulatory role.

I 273 G-PROTEIN AND TYROSINE KINASE REGULATION OF PHOSPHOINOSITIDE 3-KINASE

Peter Thomason and C.Peter Downes, Biochemistry Department, University of Dundee, Dundee, DD1 4HN, Scotland.

Phosphoinositide 3-kinase (PI 3-kinase) is activated by many different growth factors and hormones, which fall into two catagories dependent on the class of receptor they activate. Thus, both G-protein coupled receptors and receptors possessing endogenous tyrosine kinase activity have been shown to activate PI 3-kinase in cells of haematopoietic lineage, whereas in other cell types it seems that only receptor tyrosine kinases are concerned with regulation of PI 3-kinase.

We have investigated the regulation of PI 3-kinase in rat 1 fibroblasts and in human platelets. PI 3-kinase activity is regulated by PDGF in rat 1 fibroblasts, but is not regulated by thrombin. However, thrombin is known to activate PI 3-kinase in platelets, and here we show that PI 3-kinase in platelet cytosol is sensitive to GTPγS, indicating the involvement of a GTP-binding regulatory protein. We have used phosphopeptides derived from the kinase insert region of the PDGF β receptor to isolate platelet cytosolic PI 3-kinase, and we present data concerning the activation of this enzyme by these phosphopeptides and also by GTPγS and G-protien subunits. In addition, we have used a bacterial expression system to produce a GST-fusion protein containing the src-SH3 domain, and we present results concerning the interaction of the isolated src-SH3 domain with PI 3-kinase.

I 274 PREDICTION OF AUTOPHOSPHORYLATION SITES IN TYROSINE KINASES USING SYNTHETIC PEPTIDE SUBSTRATE MAPPING, Christoph W. Turck and Steven P. Edenson, Howard Hughes Medical Institute, University of California San Francisco, CA 94143-0724

Tyrosine phosphorylation has been shown to be a key step in the regulation of several cellular events including signal transduction mechanisms of stimulated growth factor receptors. Upon ligand activation growth factor receptors of the tyrosine kinase family undergo autophosphorylation on specific tyrosine residues and interact with several cellular signalling proteins. The association of the tyrosine kinase receptors with these cellular proteins is believed to be mediated by definable regions in the cytoplasmic domain of the receptor requiring a phosphorylated tyrosine residue in a specific sequence context. Methods that would allow the mapping of tyrosine phosphorylation sites would therefore greatly enhance the analysis of growth factor receptor signalling mechanisms.

We have developed a method that uses synthetic peptides encompassing all tyrosine residues of a tyrosine kinase by employing them as substrates in in vitro kinase reactions with γ-^{32}P-ATP and the respective tyrosine kinase. Tyrosine phosphorylated peptides are identified as radioactive spots after thin layer chromatography. We have applied this method to several receptor as well as cytoplasmic tyrosine kinases. In the case of the mouse PDGF β receptor we have identified 17 tyrosine containing peptides derived from the cytoplasmic domain of the protein that are phosphorylated. These peptides include 7 out of 8 tyrosines that are known autophosphorylation sites in vivo as determined by peptide mapping and mutational analyses. Furthermore two peptides that are known to contain tyrosines that are not phosphorylated in vivo did not give a signal with our method. Our method greatly simplifies the mapping of autophosphorylation sites in tyrosine kinases and provides a valuable tool in the analysis of signalling mechanisms involving these proteins.

I 275 RECEPTORS AND SIGNALING MECHANISMS IN MITOGENIC RESPONSE TO INSULIN AND GROWTH HORMONE IN A SPONTANEOUS MURINE T-CELL LYMPHOMA (LB). B. Ursø[1], L. M. Graves[2], S. Giorgetti[3], E. Van Obberghen[3], D. Naor[4] and P. De Meyts[1]. [1]Hagedorn Research Institute, 2820 Gentofte, Denmark. [2]Dept. of Pharm., U. of Washington, Seattle, WA 98195. [3] INSERM U. 145, Faculte de Médecine, Nice, France. [4]Lautenberg Center, Hebrew University, Jerusalem 91010, Israel.

Insulin is a hormone that elicits a variety of metabolic actions, however it also stimulates cell division under specific circumstances *in vivo* and when transfected *in vitro*. It induces its effects through the insulin receptor, which possesses tyrosine kinase activity. On most cells a related receptor, the insulin-like growth factor I (IGF-I) receptor, is also present, the role of which is thought to be primarily mitogenic. These two receptors are known to form hybrids, the function of which is not yet known, which complicates the analysis of the respective effect of each receptor. The LB cell-line has no IGF-I receptors and approximately 3000 insulin receptors, and is therefore an ideal model for analysis of the insulin effect (Pillemer et al. Int. J. Cancer, 50:80-85, 1992). Insulin is a strong mitogen to these cells, analysed by stimulation of DNA synthesis. Growth hormone (GH) is a even stronger mitogen, inducing its effect through a pure GH receptor. The cells possess approximately 600 GH receptors. Some of the signaling pathways activated by insulin and GH were analysed.

Mitogen activated protein kinase MAPK1 and 2 were identified in LB cells by Western blotting. The activation of MAPK1 and 2 by insulin and GH was only at most two fold the basal value, measured in either cell extracts or in immunoprecipitates, with anti MAPK antibodies, using myelin basic protein as a substrate. This activation was rapidly induced (1-5 min), and declined after 20-30 min. The difference in potency between insulin and growth hormone in stimulating the DNA synthesis was not reflected in a difference in stimulating MAPK. In contrast insulin stimulated phosphatidylinositol-3 kinase (PI3-K) 5-7 fold. The cells were stimulated *in vivo* and the activity of PI3-K was measured *in vitro* after immunoprecipitation, using phosphatidylinositol as substrate (Giorgetti et al., Eur. J. Biochem., 207:599-606, 1992). PI3-K was not stimulated by growth hormone. Insulin-stimulatable PI3-K activity was present in precipitates with anti p85 PI3-K subunit antibody (where it was highest) as well as with anti insulin receptor substrate 1 (IRS-1) or anti phosphotyrosine antibody precipitates, indicating the association of IRS-1 and PI3-K and the tyrosine phosphorylation of at least one of them.

I 276 INTERACTIONS BETWEEN THE PDGF RECEPTOR β SUBUNIT-ASSOCIATED PROTEINS: rasGAP SUPPRESSES THE PLCγ -DEPENDENT BRANCH OF MITOGENIC SIGNALING,

Mindaugas Valius and Andrius Kazlauskas, National Jewish Center for Immunology and Respiratory Medicine, Denver CO 80206

Activation of the PDGF Receptor β subunit [PDGFR] via binding of PDGF engages the receptor's intrinsic tyrosine kinase activity, the receptor undergoes tyrosine phosphorylation, enabling stable association of a group of SH2-containing signaling enzymes. Some of these receptor-associated proteins (phosphatidylinositol 3 kinase [PI3K] and phospholipase Cγ 1 [PLCγ]) are necessary and sufficient for PDGF-dependent DNA synthesis. To test the hypothesis that the proteins that associate with the PDGFR may play a negative role in mitogenic signal relay, we constructed a series of PDGFR mutants that associated with PLCγ; PLCγ and one additional protein (ras GTPase activating protein [GAP], PI3K or SH2-containing phosphotyrosine phosphatase [Syp]); or two additional proteins. The ability of this group of mutants to signal was compared with the wild type PDGFR β subunit, that associates with all four of these signaling molecules.

The amount of PLCγ that associated with the receptor was unaffected by binding of any combination of proteins, and all receptors were kinase active. Analysis of PLCγ activation in intact cells showed that the combination of PLCγ and GAP nearly completely prevented activation of PLCγ, whereas the combination of PLCγ and PI3K slightly reduced PLCγ activation. PDGFRs with all other combinations of the associated proteins activated PLCγ to wild type levels. PDGF-induced PLCγ tyrosine phosphorylation was greatly reduced in cells expressing PDGFR mutants that associated with both PLCγ and GAP. Thus GAP, or some other protein that associates with the receptor via the GAP binding site, prevents PDGF-dependent PLCγ tyrosine phosphorylation. Finally, we found that the PDGFR mutant that associated with both PLCγ and GAP was unable to initiate DNA synthesis.

These studies demonstrate a complex interaction between the various PDGFR-associated proteins, and reveal that some of the proteins that associate with the PDGFR antagonizes PLCγ tyrosine phosphorylation, its enzymatic activity, and subsequent mitogenic signaling.

I 277 IDENTIFICATION OF THE HIGH AFFINITY BINDING SITE OF TGFα FOR THE CHICKEN EGF RECEPTOR USING EGF/TGFα CHIMERAS,

E. Joop Van Zoelen, Roel H. Kramer and Monique L.M. Van De Poll, Department of Cell Biology, University of Nijmegen, 6525 ED Nijmegen, The Netherlands.

Human epidermal growth factor (hEGF) and human transforming growth factor (hTGF)-α are structurally related growth factors, which share however relatively little sequence homology. They both exert their biological effects by binding to the cell surface EGF receptor. hEGF and hTGFα bind with similar affinity to the human EGF receptor, but hEGF binds with approximately a hundred-fold lower affinity to the chicken EGF receptor than hTGFα. To map the region in hTGFα that confers its ability to bind with high affinity to the chicken EGF receptor, six hybrids of hEGF and hTGFα were constructed by exchanging domains bordered by the conserved cysteine residues three, four, and six. Activity of the expressed chimeric proteins was determined by their ability to compete with ^{125}I-mEGF for binding to NIH-3T3 cells transfected with human EGF receptor. Subsequent binding competition studies on NIH-3T3 cells transfected with the chicken EGF receptor showed that chimeras carrying TGFα sequences C-terminal from the sixth cysteine have a high affinity for this receptor, similar to hTGFα. In contrast, chimeras with EGF sequences in this C-terminal domain have only low binding affinity, similar to hEGF. It is concluded that the C-terminal linear region of hTGFα (amino acids 44-50) is important for its high affinity interaction with the chicken EGF receptor.

The present investigations are supported by a grant from the Dutch Cancer Society.

I 278 REGIONS OF GROWTH HORMONE RECEPTOR REQUIRED FOR ITS INTERACTION WITH JAK2 TYROSINE KINASE.

Joyce A. VanderKuur, Nils Billestrup+, Gunnar Norstedt*, and Christin Carter-Su, Department of Physiology, University of Michigan, Ann Arbor, MI 48109-0622; +Hagedorn Res. Lab., Gentofte, Denmark; *Karolinska Ins., Huddinge, Sweden.

In previous work, we have shown that the growth hormone (GH) receptor associates with and activates JAK2, a recently identified cellular tyrosine kinase. To gain insight into the region(s) of the GH receptor (GHR) involved in the GHR-JAK2 interaction, various rat liver GHR mutants were examined for their ability to associate with JAK2 and stimulate JAK2 tyrosyl phosphorylation. The mutant GHRs include GHR_{1-454} which lacks the C-terminal half of the GHR cytoplasmic domain, GHR_{1-380} which lacks 75% of the cytoplasmic domain, GHR_{1-294} which lacks all but 5 amino acids of the cytoplasmic domain, and GHR_{4P-A} in which 4 prolines (300, 301, 303, and 305) in a proline-rich region just proximal to the transmembrane domain were mutated to alanines. The ability of JAK2 to associate with the mutated GHRs was determined by immunoprecipitating GH-GHR complexes and associated proteins with anti-GH antibody (αGH) and immunoblotting the precipitated proteins with anti-JAK2 antibody (αJAK2). JAK2 was found to associate with wild-type GHR (GHR_{1-638}), GHR_{1-454} and GHR_{1-380}, but not GHR_{1-294} or GHR_{4P-A}. To examine whether GH binding to the mutated GHR could stimulate JAK2 tyrosyl phosphorylation (an indication of JAK2 kinase activity), CHO cells expressing the various GHR mutants were incubated with GH. JAK2 was immunoprecipitated with αJAK2 and immunoblotted with antiphosphotyrosine antibody (αPY). GH-dependent tyrosyl phosphorylation of a protein migrating with a Mr (130,000) appropriate for JAK2 was observed in cells expressing GHR_{1-638}, GHR_{1-454} and GHR_{1-380}, but not GHR_{1-294} or GHR_{4P-A}. When GH-GHR complexes were immunoprecipitated using αGH and immunoblotted with αPY, GHR_{1-638} and GHR_{1-454} but not GHR_{1-294} or GHR_{4P-A} were tyrosyl phosphorylated in response to GH, indicating the N-terminal half of the cytoplasmic domain of GHR contains tyrosyl phosphorylation sites for JAK2. These results indicate that the N-terminal quarter of the cytoplasmic domain of the GHR, possibly the portion of the receptor containing the proline-rich region, is required for association with and activation of JAK2. Association of JAK2 with the proline-rich region would be consistant with previous findings that deletion or mutation of this region in GHR and other members of the cytokine receptor family that signal through JAK2 leads to loss of function.

I 279 MIS RECEPTOR MEDIATED INHIBITION OF EGFR AUTO-PHOSPHORYLATION

Tong Wen Wang, Melinda A. Maggard, Paresh C. Shah, Elizabeth A. Catlin, David T. MacLaughlin, Patricia K. Donahoe Pediatric Surgical Research Laboratories, Massachussetts General Hospital, Boston, MA 02114

MIS (Mullerian Inhibiting Substance) and EGF (Epidermal Growth Factor) are natural antagonists in several physiological processes. Partially purified bovine and recombinant human MIS have been shown to inhibit EGF tyrosine kinase receptor autophosphorylation in a tumor cell line, A431 (vulvar epithelial carcinoma). MIS has also been shown to cause growth arrest in A431 at the G1 stage of the cell cycle. Purified holo-MIS, however, does not cause growth arrest, or inhibition of EGF receptor auto-phosphorylation. This implies that MIS may require proteolytic cleavage for bio-activity. When MIS is plasmin cleaved into a 110kDa amino terminal and a 25kDa carboxy-terminal fragment, it can inhibit A431 growth. Recent data identified the carboxy-terminus (rhMIS-C) as the active component, causing dose-dependent regression of the fetal rat Mullerian duct. RhMIS-C has now been shown to cause growth inhibition of A431 as well. Herein we report that both plasmin cleaved MIS and the purified MIS C-terminus can inhibit EGF receptor auto-phosphorylation. To further understand that this inhibition is mediated via a MIS specific receptor, and not by competitive MIS binding to the EGF receptor, we carried out chemical cross-linking assays on A431 with ^{125}I labelled plasmin cleaved MIS and/or purified MIS C-terminal fragment. We identified a 70-80 kDa protein that binds to MIS specifically on the A431 cell surface. Similar studies performed on another tumor cell line, OM431 (ocular melanoma), generated a more intense band with a similar molecular weight, suggesting that OM431 contains a higher copy number of MIS receptor. Preliminary data indicates that the rhMIS-C induced inhibition of auto-phosphorylation may be mediated via a protein-tyrosine phosphatase (PTPase). Phosphatase inhibition by vanadate blocks the rhMIS-C mediated effects. Current efforts are directed at cloning the MIS receptor in the A431 cell line, to elucidate the mechanisms involved in the MIS receptor mediated inhibition of EGF receptor auto-phosphorylation.

I 280 COLLAGEN ACTIVATES PLATELETS THROUGH A TYROSINE-KINASE DEPENDENT PATHWAY, Steve P Watson, Alastair Poole, Judith Asselin and Robert Blake, Department of Pharmacology, Mansfield Road, Oxford, OX1 3QT, U.K.

Collagen is an adhesion molecule important in the primary activation of platelets during haemostasis. The molecular basis of collagen-induced platelet activation is not known. Platelets express an apparent redundancy of binding sites for collagen which lack the seven transmembrane structure characteristic of G protein-coupled receptors and do not contain intrinsic tyrosine or serine/threonine kinase activity. In the present study, we show that collagen-induced activation of phospholipase C (PLC) and mobilisation of Ca^{2+} are inhibited completely by the nonselective inhibitor of protein kinases, staurosporine, and by a selective inhibitor of protein tyrosine kinases, ST271. Similar results are obtained with immune complexes (which induce activation through cross-linking of the Fcγ-RII receptor) and wheat germ agglutinin (which induces cross-linking of glycoproteins) but not by G protein receptor agonists. Collagen, WGA and immune complexes, but not thrombin, induce marked tyrosine phosphorylation of 38 and a 70 kDa proteins independent of protein kinase C, Ca^{2+} mobilisation and aggregation. The 70 kDa protein has been tentatively identified as the non-receptor tyrosine kinase, *syk*, suggesting that it may mediate many if not all of the responses associated with collagen-induced platelet activation.

I 281 ACUTE-PHASE RESPONSE FACTOR (APRF) IS RAPIDLY ACTIVATED BY IL-6 AT THE POSTTRANSLATIONAL LEVEL AND IMMUNOLOGICALLY RELATED TO THE ISGF3 FAMILY OF TRANSCRIPTION FACTORS,
U. M. Wegenka, C. Lütticken, J. Yuan, J. Buschmann, P. C. Heinrich and F. Horn, Institut for Biochemistry, RWTH Aachen, Pauwelstrasse 30, D-52057 Aachen, Germany

IL-6 is the major inducer of acute-phase protein synthesis in liver. Furthermore, IL-6 is involved in the differentiation of B cells, T cells, macrophages and neuronal cells and functions as growth factor for certain myelomas and hybridomas. Biological functions of IL-6 are mediated through a signal-transducing component of the IL-6 receptor, gp130, which is associated with the ligand binding subunit, gp80. The IL-6 receptor has no intrinsic kinase activity and the signal transduction pathways leading to gene activation are unresolved so far.
Recently, we reported that a nuclear factor called APRF (acute-phase response factor) is rapidly activated after IL-6 stimulation in vivo and in vitro. We now show that APRF is also activated by the cytokines LIF, OSM, IL-11 and CNTF which are known to share the signal-transducer gp130 with the IL-6 receptor system. APRF is tyrosine phosphorylated in response to IL-6 and is detectable in the cytoplasm within minutes. APRF therefore represents an excellent candidate for the transduction of IL-6 signals to the nucleus.
The DNA binding sites for APRF were carefully investigated and found to match the palindromic sequence $TT^{C}/_{A}CNG^{G}/_{T}AA$. Binding sites for APRF are crucial for the IL-6 induction of acute-phase genes such as α_2-macroglobulin, haptoglobin and fibrinogen but were also identified in the IL-6 response elements of IL-6-induced immediate-early genes. The same binding motif was shown to be bound by IFNγ-activated members of the ISGF3 family of transcription factors. We purified APRF to homogeneity and present data that APRF is distinct from ISGF3 transcription factors but is most likely a member of a growing family of ISGF3-related proteins.

I 282 THE ROLE OF TYROSINE KINASE RECEPTORS IN ZEBRAFISH EMBRYOGENESIS, Ellen T. Wilson, Barbara Hug and David J. Grunwald, Dept. of Human Genetics, Eccles Inst. of Genetics, Univ. of Utah, SLC, UT 84112
Our goal is to characterize the expression of tyrosine kinase receptors (TKRs) in the zebrafish embryo and to test directly how the functions of these receptors contribute to early embryonic development. Our work is motivated by the fact that, while it is clear that tyrosine kinase receptors regulate proliferation and differentiation of many cell types, relatively little is known about their contribution to early stages of embryogenesis. We have recently identified six tyrosine kinase receptors present in the early zebrafish embryo at the times when the primary tissues first arise, during gastrulation and neurulation. To determine how the receptors are utilized during embryogenesis, we have examined the developmental pattern of their expression by whole-mount hybridization *in situ*. One example of a receptor that we deem suited for further functional analysis is Hepatocyte Growth Factor Receptor (HGFR). HGFR's expression is restricted to incipient neural tissue at the time of neural plate induction and can be detected throughout the embryonic nervous system as differentiation progresses. A possible role for Hepatocyte Growth Factor (also called Scatter Factor) in neural induction in the chick was recently proposed by Stern and Ireland. To reveal what developmental role is normally played by the zebrafish TKRs, we plan to use 'dominant negative' forms of the receptors to experimentally inhibit their signaling activity. Microscopic and histological analyses will be used to assess the developmental effects associated with loss of receptor function.

I 283 IDENTIFICATION AND CHARACTERISATION OF FOCAL ADHESION KINASE IN VASCULAR SMOOTH MUSCLE,
Lynn Wilson and Martin J. Carrier, Yamanouchi Research Institute, Littlemore Hospital, Oxford OX4 4XN, U.K..
Cell surface association with the extracellular matrix represents an important aspect of cellular behaviour, controlling apparently such diverse functions as differentiation, proliferation and migration. Recently a focal adhesion-associated kinase, termed focal adhesion kinase (FAK) has been identified in mouse and human fibroblasts. The structure of this kinase appears to be unique and may well represent a novel class of kinases. We have examined the role of FAK in human and mouse vascular smooth muscle. These cells are known to undergo a phenotypic change, followed by migration and subsequently proliferation, during atherogenesis, and hence represent ideal cells for the study of focal adhesion organisation and FAK in particular. Using a panel of RNA templates from smooth muscle cells stimulated from quiescence with either IL-1β, PDGF or serum we have identified FAK specific mRNA (confirmed by sequencing). FAK did not appear to be transcriptionally regulated under these growth conditions. The presence of focal adhesions generated in response to adherence to extracellular matrix components, such as fibronectin, has been confirmed by immunofluoresence using anti-vinculin and anti-talin antibodies. Phosphotyrosine containing proteins also co-localised to adhesion plaques under these conditions. We have shown that using anti-FAK immunoprecipitation followed by *in-vitro* kinase assay, that FAK is activated over an approximately 60 minute time period following plating onto fibronectin.
This is the first demonstration of the presence of FAK in human and mouse vascular smooth muscle. FAK in these cells can be activated by phosphorylation and is associated with adhesion plaques. Finally we believe that the study of FAK, and FAK associated events, in vascular smooth muscle may provide a novel insight into the pathogenesis of atherosclerosis.

I 284 REGULATION OF THE P56^{LCK} SH2 DOMAIN BY PHOSPHORYLATION OF SER 59 IN THE UNIQUE N TERMINAL REGION OF LCK, David G. Winkler, Insil Joung, Indal Park, Tae Ue Kim, and Jeakyoon Shin, Division of Tumor Virology, Dana Farber Cancer Institute, Boston, MA, 02115

Ser59 in the unique N-terminal region of p56lck is phosphorylated during T cell activation. This phosphorylation is mimiced by mutation of Ser59 to Glu59. The Ser59Glu lck induces the tyrosyl-phosphorylation of unique intracellular proteins upon CD4 crosslinking. This mutant has similar affinity to CD4 and similar kinase activity as wild type p56lck. A GST fusion protein containing the lck SH2 domain, the SH3 domain, and a portion of the unique N-terminal region including the Ser 59 phosphorylation site has a different binding specificity for phosphotyrosyl-proteins when compared to SH2 domain alone.The *phosphotyrosyl-protein* binding specificity reverts back to that of the SH2 domain alone if the unique N-terminal region is deleted or if Ser 59 is mutated to Glu 59. These results suggest that modification of Ser59 regulates the function of p56lck by controlling binding specificity of the SH2 domain.

I 285 HEMATOPOIETIC CELL PHOSPHATASE (HCP) ASSOCIATES WITH THE IL-3 RECEPTOR β CHAIN AND DOWN-REGULATES IL-3 INDUCED TYROSINE PHOSPHORYLATION AND CELL PROLIFERATION, Yi, T., L. Alice, G. Krystal and J. N. Ihle. Department of Cancer Biology, The Cleveland Clinic Foundation, Cleveland, OH44195, The Terry Fox Laboratory, British Columbia Cancer Agency, British Columbia, Canada V5Z1L3 and Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38105

Hematopoietic Cell Phosphatase (HCP) is a tyrosine phosphatase with two src-homology-2 (SH2) domains that is predominately expressed in hematopoietic cells, including cells whose growth is regulated by interleukin-3 (IL-3). The potential effects of HCP on IL-3 induced protein tyrosine phosphorylation and growth regulation were examined to assess the role of HCP in hematopoiesis. Our studies demonstrate that, following ligand binding, HCP specifically associates with the β chain of the IL-3 receptor through the amino terminal SH2 domain of HCP, both *in vivo and in vitro*, and can dephosphorylate the receptor chain *in vitro*. The effects of increasing or decreasing HCP levels in IL-3 dependent cells were assessed with dexamethasone inducible constructs containing an HCP cDNA in sense and anti-sense orientations. Increased HCP levels were found to reduce the IL-3 induced tyrosine phosphorylation of the receptor and to suppress cell growth. Conversely, decreasing the levels of HCP increase IL-3 induced tyrosine phosphorylation of the receptor and increased the cell growth rate. These results support a role for HCP in the regulation of hematopoietic cell growth and begin to provide a mechanistic explanation for the dramatic effects that the genetic loss of HCP, which occurs in *motheaten (me)* and *viable motheaten (me^v)* mice, has on hematopoiesis.

Poster Session III

I 300 **8-METHOXYPSORALEN FORMS PHOTOADDUCTS WITH CELLULAR PHOSPHOLIPIDS IN CULTURED MELANOGENIC CELLS AND 8-METHOXYPSORALEN-FATTY ACID PHOTOADDUCTS ACTIVATE PROTEIN KINASE C.** Frank A. Anthony, Henry M. Laboda, John C. Dowdy, and Mark E. Costlow, Advanced Product Research, Schering-Plough HealthCare Products, Memphis, TN 38151

Psoralen in combination with UVA (PUVA) is effective treatment for psoriasis, mycosis fungoides, and vitiligo. Formation of covalent 8-methoxypsoralen (8-MOP) photoadducts with cell structures is thought to be important for PUVA action. DNA is believed to be a primary target for psoralen photoattachment, but on skin, psoralen mostly forms photoadducts with proteins and lipids. We studied 8-MOP covalent photobinding to lipids in cultured S91 mouse melanoma cells and normal human melanocytes (NHM). Cells were treated with UVA and 50 nM [^{3}H]8-MOP at 4°C with or without unlabeled excess 8-MOP. Separation of chloroform/methanol cell extracts by TLC revealed the presence of [^{3}H]8-MOP-lipid photoadducts. Binding was selective for phospholipids and partially competitive with excess unlabeled 8-MOP. The 8-MOP phospholipid photoadducts favored PI/PS over PC or PE based on the relative content of phospholipid in S91 cells. [^{3}H]8-MOP phospholipids were isolated, digested with phospholipase A_2, C, or D, and examined by TLC. [^{3}H]8-MOP bound covalently to the head group or the 2-position of the acyl chain. 8-MOP- and dihydrotrimethylpsoralen-fatty acid photoadducts prepared in vitro from linoleic, linolenic, and arachidonic acids activated protein kinase C (PKC) from S91 melanoma cells. These photoadducts substituted for diacylglycerol (DAG) to activate PKC. 8-MOP-phospholipid photoadducts, 8-MOP, and UV-irradiated arachidonic acid were inactive towards PKC. These data suggest 8-MOP-phospholipids or their metabolites may be important in the biological actions of PUVA through PKC activation.

I 301 DISHEVELLED MEDIATES NOTCH SIGNALLING TO PRODUCE SENSORY ORGANS ON THE ANTERIOR MARGIN OF THE DROSOPHILA WING BLADE, Axelrod, J.D. and Perrimon, N., Department of Genetics and Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115

Notch(N), the Drosophila homologue of the human TAN-1 protein, is a multifunctional transmembrane receptor molecule involved in a variety of cell-cell signalling events. N mediated signal transduction is poorly understood. On the anterior margin of the wing, N is required to produce bristles that function as sensory organs. Differentiation of bristle precursors, the sensory mother cells (SMCs), can be monitored using the enhancer trap A101, which reflects neuralized gene expression. In N mutants, SMCs do not develop and bristles are lost from the margin. zest-white3 (zw3), the Drosophila homolog of the human ser/thr kinase glycogen-synthase kinase 3, appears to function downstream of N. zw3 activity suppresses expression of the achaete-scute complex(AS-C), which is required for differentiation of SMCs. Activation of N may therefore block zw3 activity, allowing AS-C expression and bristle differentiation. We have demonstrated that dishevelled(dsh), a novel protein with homologs in vertebrates, is required to mediate this activity of N on the anterior wing margin. Bristles fail to differentiate in clones of wing tissue mutant for dsh, and instead appear in wild type tissue adjacent to the clone, demonstrating that dsh is required for bristle differentiation and functions cell autonomously. In contrast, overexpression of dsh from a heat shock promoter induces ectopic SMC differentiation, and results in ectopic bristle formation. We will present evidence that dsh acts downstream of N, and directly mediates N activity, thereby allowing activation of the AS-C, and thus SMC differentiation. Furthermore, we will present experiments that identify other components in the signalling pathway, including possible ligands for N. Interestingly, this pathway appears to reiterate one active in determining segmentation in the embryo.

I 302 OXIDATIVE STRESS STIMULATES MITOGEN-ACTIVATED PROTEIN KINASES AND VASCULAR SMOOTH MUSCLE CELL GROWTH, Arnold S. Baas, Jennifer L. Duff, Bradford C. Berk, Department of Cardiology, Emory University School of Medicine, Atlanta, GA 30322

Oxidative stress has been shown to stimulate tyrosine phosphorylation by activating protein tyrosine kinases and inhibiting protein tyrosine phosphatases. Because oxidative stress is mitogenic for vascular smooth muscle cells (VSMC), we hypothesized that oxygen radicals should stimulate growth factor activated protein kinases such as the mitogen-activated protein kinase (MAPK). To cause oxidative stress, we utilized H_2O_2 and the O_2- generating napthoquinone LY83583. As compared to 0.1% serum, 10 ng/ml PDGF and 0.3 μM LY83583 stimulated maximal increases in 3H-thymidine incorporation of 75% and 42% respectively. Peak 3H-thymidine incorporation occurred at 18-24 h after exposure to PDGF and 6-24 h after exposure to LY83583. To study regulation of MAPK by H_2O_2 and LY83583, we utilized Western blot analysis to detect the "band shift" of the phosphorylated p42 and p44 MAPK. There was a concentration dependent increase in MAPK activation by LY83583 (peak at 0.3 μM), but not by H_2O_2. The time course for activation was similar to that observed with PDGF and angiotensin II: LY83583 activated both p42 and p44 MAPK at 5 min with return to baseline by 20 min. To determine why H_2O_2 failed to activate MAPK, we studied 3CH134, a protein tyrosine phosphatase with high activity for MAPK. 3CH134 is transcriptionally regulated and its mRNA levels were increased by 100 μM H_2O_2 at 1 hour, but not by 0.3 μM LY83583. In addition, 100 μM H_2O_2 inhibited 1 nM angiotensin II-induced MAPK activation. These findings suggest 3 mechanisms for regulation of MAPK by oxidative stress: activation of protein tyrosine kinases and/or inhibition of protein tyrosine phosphatases by superoxide, and the induction of the phosphatase 3CH134 by H_2O_2. These data demonstrate that different types of oxidative stress in VSMC cause different effects on growth related signal transduction events.

I 303 INVESTIGATION OF THE ROLE OF G-PROTEINS IN PROTEIN TRANSPORT IN THE MALARIA INFECTED ERYTHROCYTES

Richa Behari and Kasturi Haldar, Department of Microbiology and Immunology, Stanford University School of medicine, Stanford CA 94035-5402

The malarial parasite *Plasmodium falciparum* invades and develops in mature erythrocytes. The secretory pathway of the early, intraerythrocytic stage parasite is simple and to date, its Golgi complex is found to contain only two compartments. Yet it displays unique features which include novel membrane dynamics of the Golgi and the export of tubovesicular membranes into the erythrocyte cytosol. In the later intraerythrocytic stages, apical, secretory organelles (required for the next round of parasite invasion into the red cell) develop, presumably from the parasite's Golgi. Small G (GTP binding)-proteins of the "rab family" are known to play a central role in secretory membrane transport in cells. The objective of this project is to identify and characterize parasite encoded "rab like" G-proteins which regulate transport properties of the malarial Golgi.

I 304 **GROWTH FACTOR-DEPENDENT ACTIVATION OF THE GLUCOSE TRANSPORTER ON A BONE MARROW-DERIVED CELL LINE, 32D, IS MEDIATED THROUGH RECEPTOR-ASSOCIATED PHOSPHORYLATION INDEPENDENTLY OF SIGNAL TRANSDUCTION TO THE NUCLEUS** Michael V. Berridge and An S. Tan Malaghan Institute of Medical Research, Wellington School of Medicine, Wellington, New Zealand.

Glucose transport across the plasma membrane occurs via a family of 'facilitative' glucose transporter molecules which passively shift glucose against it's concentration gradient towards equilibrium. In insulin-dependent tissues, increased glucose transport occurs by recruitment of stored GLUT-4 transporter molecules, and in the longer term by GLUT-4 gene expression. However, in these and in most other tissues, basal glucose transport occurs via GLUT-1. Involvement of insulin and growth factors in the regulation of GLUT-1 activity is however controversial. In this study we show that the hemopoietic growth factor, interleukin-3 (IL-3), regulates glucose transport by maintaining the affinity of the transporter for glucose. (K_m=1.23±0.05, n=7). Withdrawl of IL-3 resulted in reduced affinity for glucose (K_m=2.96±0.28, n=4) at 1 hour, at which time there was no change in V_{max}, and glucose transporter numbers on the cell surface remained unaltered as determined by cytochalasin B binding to plasma membrane fractions. Changes in glucose transport could be detected within 3 minutes of IL-3 treatment/withdrawl. Inhibition of protein tyrosine kinases with genistein or erbstatin, or of protein kinase C (PKC) with calphostin C, resulted in rapid loss of ability to take up glucose. Conversely, the tyrosine phosphatase inhibitor, sodium vanadate, stimulated glucose transport by 50-80%. The effects of IL-3 on glucose uptake were not dependent on DNA synthesis or respiration as determined with mitomycin C and sodium azide. These results show that IL-3 is involved in continuous activation of glucose transporter molecules via signal transduction mechanisms that involve tyrosine phosphorylation and protein kinase C, and that this activation is not dependent on signal transduction to the nucleus.

I 305 CHARACTERIZATION OF VAV SH2-BINDING PROTEINS IN T AND B LYMPHOCYTES.

Nathalie Bonnefoy-Berard[1], Shulamit Katzav[2], Erich Gulbins[1], K. Mark Coggeshall[1] and Amnon Altman[1], Division of Cell Biology, La Jolla Institute for Allergy and Immunology[1], Lady Davis Institute, Jewish General Hospital, Montreal, Quebec H3T 1E2, Canada[2]

Ras proteins function as key molecular switches that regulate cellular growth and differentiation responses initiated by receptor or non-receptor tyrosine kinases. They are activated by guanine nucleotide exchange factors (GEFs) that exchange bound GDP for GTP. We recently identified Vav, the 95-kDa product of the *vav* protooncogene, which is exclusively expressed in hematopoietic cells, as a Ras-specific GEF activated by two independent pathways, *i.e.*, antigen receptor-coupled protein tyrosine kinases (PTKs), or diglycerides. Here, we evaluated by immunoblotting and *in vitro* kinase assays the binding of proteins from activated B or T cell lysates to Vav SH2-GST fusion proteins immobilized on glutathione-coupled agarose beads. Specificity controls included the GST protein alone or an inactive Vav SH2 fusion protein generated by site-directed mutagenesis of residues known to be critical for SH2 function. Partially overlapping but distinct profiles of Vav SH2-associated proteins were detected by anti-phosphotyrosine (PTyr) immunoblotting in T *vs.* B cells. A PTyr-containing 71-kDa protein, which was identified as the ZAP-70 PTK, was readily detected in activated T cell lysates. B cells lysates contained a major ~68-kDa protein which was highly phosphorylated 30 seconds after surface immunoglobulin ligation. This protein was not recognized by antibodies specific for ZAP-70 or a related, B cell-expressed PTK, $p72^{syk}$. Two additional PTyr-containing proteins of ~100 and ~110 kDa were detected in activated cell lysates from both T and B cells. *In vitro* kinase reactions of Vav SH2-bound proteins from activated B or T cells revealed kinase activity which was stimulated by anti-receptor antibodies. Phosphoamino acid analysis indicated that the Vav SH2 domain itself was a substrate for serine/threonine phosphorylation. The identity and properties of the Vav SH2-bound kinase(s) are presently under investigation.

I 306 INSULIN- AND EGF-INDUCED ACTIVATION OF P21RAS AND ERK, Johannes L. Bos, Gijsbertus J. Pronk, and Alida M. M. de Vries-Smits, Pascale C. van Weeren and Boudewijn M.Th. Burgering. Laboratory for Physiological Chemistry, Utrecht University, 3521 GG Utrecht, The Netherlands.

A14 cells are NIH3T3 cells overexpressing the human insulin receptor. Insulin treatment as well as EGF treatment results in a dramatic increase in p21rasGTP [Burgering et al. (1991) *EMBO J.*, **10**, 1103], as a consequence of increased nucleotide exchange activity [Medema et al. (1993). *Mol. Cell. Biol.*, **13**, 155]. We have investigated the mechanism by which insulin and EGF activate p21ras and Erk2. First, after insulin treatment Shc [Pronk et al. (1993) J. Biol. Chem. **268**, 5748] and IRS1 are phosphorylated on tyrosine. Both proteins associate with Grb2 as well as with the p21ras nucleotide exchange factor mSos, although the association of mSos with IRS1 is barely detectable. This result suggests a dual pathway to activate p21ras, with a prominent role for Shc. After EGF treatment association between both mSos and Shc, and mSos and the EGF receptor is observed. Secondly, insulin-induced activation of Erk2 is mediated by p21ras, whereas EGF-induced activation of Erk2 is mediated by at least two different pathways. One pathway is sensitive to inhibition by the interfering mutant p21rasasn17 and is thus mediated by p21ras; the other pathway is sensitive to EGTA treatment, suggesting calcium dependency. In Swiss3T3 cells this calcium dependent pathway is not observed. Instead, a pathway sensitive to TPA-pretreatment is found, suggesting PKC-dependency [Burgering et al. *Mol. Cell. Biol.* in press]. Thirdly, 8-bromo-cAMP inhibits both insulin and EGF-induced activation of Erk2 and Raf1, but not of p21ras, suggesting inhibition of the pathway between p21ras and Raf1. Using 8-bromo-cAMP as inhibitor we have identified several targets of the Raf1/Erk2 pathway, including mSos [Burgering et al. (1993) *EMBO J.* in press].

I 307 MODULATION OF ILEUM CHOLINERGIC MUSCARINIC RECEPTORS BY RECOMBINANT INTERFERON γ. Maria M.E. de Bracco, Leonor Sterin-Borda, Martin Rodriguez and Enri Borda. CEFYBO-CONICET and IlHEMA, Academia Nacional de Medicina, P. de Melo 3081, 1425 Buenos Aires, Argentina.

Recombinant rat interferon γ stimulated the contractility of isolated rat ileum at doses of 4-12 units/ml. The reaction was abolished by preincubation of interferon γ with monoclonal anti rat interferon γ. Interaction with the gut's muscarinic cholinoceptors was necessary, as treatment of the tissue with atropine prevented the contractile response of the ileum. Furthermore, interferon γ increased the affinity of carbachol for the cholinoceptors and did not change its maximum effect. In contrast to the action of carbachol on this tissue, the effects of interferon γ were regulated by pertussis toxin-sensitive G proteins and were blunted by treatment with phospholipase C inhibitors (2-nitro-carboxyphenyl, NN-diphenyl carbamate and neomycin). Cytoskeletal structures participated in the reaction, since cytochalasine B and colchicine treated ileum strips did not respond to interferon γ.

I 308 MOLECULAR MECHANISM OF A TISSUE SPECIFIC RAS RESPONSE IN A NEUROENDOCRINE CELL LINE, Andrew P. Bradford, Kerry E. Conrad and Arthur Gutierrez-Hartmann. Division of Endocrinology, Program in Molecular Biology, University of Colorado Health Sciences Center, Denver CO 80262.

The GTP binding protein p21 Ras has been shown to be a critical component of signaling pathways which control cell differentiation and proliferation in response to extracellular stimuli. Ras appears to function as a switch to convert the tyrosine kinase signal initiated at a transmembrane receptor to a serine phosphorylation cascade mediated by Raf, MEK and MAP kinases. However components of the pathway may be different in distinct cell types and the precise mechanisms by which common growth factor/ ras induced signals result in cell-specific responses remain to be determined. The GH_4 rat pituitary cell line represents an excellent model system to address this question since they are immortalized, highly differentiated, neuroendocrine cells which retain hormonal responses. Using a transient co-transfection assay we have demonstrated that oncogenic V-12 Ras selectively stimulates the promoter activity of an endogenous gene, rat prolactin (rPRL), whose expression is highly restricted to the pituitary cell type. Consistent with previous data in other cell types, we have shown that the Ras signal in GH_4 cells is propagated through Raf, MEK and MAP kinases. Furthermore we have identified the nuclear acceptor of the Ras signal as a member of the ets family of transcription factors and shown a functional interaction between Ras, ets-1 and the pituitary specific transcription factor GHF1/Pit1. Thus co-transfection of ets-1 and/or GHF1 in the presence of activated Ras, results in a significant, synergistic, increase in the ras response, whereas ets and/or GHF1 alone do not substantially activate the rPRL promoter. This synergistic ras response can be reconstituted in HeLa cells, which do not express GHF1 or prolactin and have low amounts of ets, by co-transfection with V-12 Ras, c-ets 1, GHF1 and a rPRL promoter reporter construct. We have mapped the Ras responsive element (RRE) of the rPRL promoter to a region, within the proximal 425 bases, which contains both a putative ets binding site and an adjacent GHF1 site. Moreover gel shift assays demonstrate that this putative RRE binds both ets and GHF1 in vitro. Interestingly the growth hormone promoter which also requires GHF1, but lacks a corresponding ets binding site, does not respond to Ras. These data indicate that a ras dependent functional interaction of a ubiquitous, ets-1, and a cell specific transcription factor, GHF1/Pit1, confers a pituitary specific response to the general ras signaling pathway.

I 309 EXTRACELLULAR SV40 ACTIVATES PRIMARY RESPONSE GENES, Walter C. Breau and Leonard C. Norkin, Department of Microbiology, University of Massachusetts, Amherst, MA 01003. SV40 induced the transcriptional upregulation of the primary response genes c-myc and c-jun within 30 minutes of exposure to growth-arrested cells, and of JE within 90 minutes. The upregulation occurred in the presence of cycloheximide and when UV-inactivated SV40 was adsorbed to cells. The effects of the protein kinase inhibitors genistein, H7, and HA1004, showed that tyrosine and serine/threonine kinases are factors in the SV40-activated signal pathways which induce the primary response genes. Furthermore, multiple signal pathways are activated by the SV40 receptor, and the individual primary response genes respond selectively to different signal pathways. SV40 did not appear to mobilize intracellular Ca^{2+}, as monitored with a sensitive digital fluorescent imaging microscope. Blocking the SV40-activated signal pathways in growth arrested cells with genistein resulted in delayed SV40 DNA synthesis. Genistein did not affect expression of SV40 T antigen mRNA. Thus, the early SV40-induced signal promotes the timely replication of SV40 DNA in growth arrested cells, probably via a direct effect on the host cell. The signal apparently induces cell competence via the activation of primary response genes. At later times, T antigen may act as a progression factor (J. Koniecki, P. Nugent, J. Kordowska, and R. Baserga, Cancer Res. 51: 1465-1471, 1991), enabling competent cells to enter S and support SV40 DNA synthesis. Antibody-induced cross-linking of MHC class I proteins also caused upregulation of c-myc and c-jun. Furthermore, the upregulation of the individual primary response genes by anti-HLA showed the same sensitivities to the kinase inhibitors as the upregulation induced by SV40. These results are consistent with the possibility that class I proteins may be involved in transmitting the extracellular SV40-induced signal, in addition to facilitating SV40 binding (W.C. Breau, W.J. Atwood, and L.C. Norkin, J. Virol. 66: 2037-2045, 1992).

I 310 **ENDOTHELINS STIMULATE TYROSINE PHOSPHORYLATION AND ACTIVITY OF p42 / MITOGEN-ACTIVATED PROTEIN KINASE IN ASTROCYTES,** Sylvie CAZAUBON°*, Peter J. PARKER*, A. Donny STROSBERG° and Pierre-Olivier COURAUD°, °ICGM, CNRS UPR 0415, 75014 Paris, France and * I C R Fund, London WC2A 3PX, United Kingdom

Endothelins (ET-1, -2, -3) display pleiotropic activities, by signalling through G protein-coupled membrane receptors. We show here that ET-1 and ET-3 stimulate within minutes the tyrosine phosphorylation of a 42-kDa protein (p42) in primary cultures of mouse embryo astrocytes. This effect, measured by anti-phosphotyrosine immunoblotting of cell extracts, was also observed in response to bradykinin, PDGF, the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate and the G protein activator fluoroaluminate. Pretreatment of cells with pertussis toxin, which inactivates Gi/Go proteins, did not affect these responses. However, down-regulation of protein kinase C completely blocked the response to phorbol ester and fluoroaluminate and at least partially impaired the ET-1-stimulated phosphorylation of p42. We have identified p42 as $p42^{mapk}$, a mitogen-activated protein (MAP) kinase, on the basis of the following data : by sequential immunoblotting with anti-phosphotyrosine and anti-MAP kinase antibodies, (i) similar kinetics are observed for p42 phosphorylation and the reduction of $p42^{mapk}$ electrophoretic mobility, likely corresponding to its tyrosine/threonine phosphorylation (de Vries-Smits et al., 1992); (ii) p42 and the shifted form of $p42^{mapk}$ co-migrate on SDS-PAGE; (iii) the MBP kinase activity of $p42^{mapk}$ is stimulated by ET-1, in parallel with the tyrosine phosphorylation of p42. In conclusion, these findings strongly suggest that endothelins can stimulate the tyrosine phosphorylation and activation of $p42^{mapk}$ in astrocytes, via pertussis toxin-insensitive G protein, protein kinase C-dependent and -independent pathways.

I 311 A GENETIC SCREEN FOR GENES DOWNSTREAM OF RAS IN *DROSOPHILA* EYE DEVELOPMENT

Henry Chang, Felix Karim, David Wassarman, Marc Therrien and Gerald Rubin. Howard Hughes Medical Institute, Department of Molecular & Cell Biology, University of California, Berkeley, CA 94720

Differentiation of the R7 photoreceptor neuron in the *Drosophila* compound eye is controlled by the Sevenless receptor tyrosine kinase and the *Drosophila* homolog of the mammalian H-Ras protein (Simon et al., 1991, *Cell* 67, 701-716). An activated form of Ras (Ras-V12) under control of the *sevenless* promoter produces ectopic R7 cells which results in a rough eye phenotype (Fortini et al., 1992, Nature 355, 559-561). The degree of roughness is graded and appears to depend on the expression level of the activated Ras construct, hence, a two-fold reduction in a gene that acts downstream of Ras may affect the degree of eye roughness.

To identify genes whose products play a critical role downstream of Ras in the Sevenless signal transduction cascade, we have used this sensitized system to screen for modifying mutations that either enhance or suppress the rough eye phenotype of activated Ras. We screened ~850,000 flies and isolated several hundred mutants that fall into several complementation groups of suppressor and enhancers. In addition to isolating mutations in genes that have been shown to be involved in Ras mediated signal transduction (e.g., Raf, MAPKK, MAPK), we hope to identify novel components in this pathway. Genetic and molecular characterization is in progress to gain further understanding of these genes.

I 312 THE MAP-2 KINASE PHOSPHORYLATION AND OLIGOMERIZATION STATES OF THE RAS NUCLEOTIDE EXCHANGE FACTOR SON OF SEVENLESS,

Andrew D. Cherniack, Jes K. Klarlund, and Michael P. Czech, Program in Molecular Medicine and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, 373 Plantation Street, Worcester, MA 01605

Son of sevenless (Sos) is a guanosine nucleotide exchange factor implicated in the activation of Ras by both the insulin and EGF signal transduction pathways. Ras appears to function by activating cellular protein kinases including MAP kinase. Sos proteins contain numerous sequence motifs in their carboxyl terminal regions which correspond to consensus sites for MAP-2 kinase phosphorylation. To examine whether these sites are substrates for MAP-2 kinase, the cDNA encoding *Drosophila* Sos (dSos) was tagged with sequences encoding the major antigenic epitope of the influenza virus hemagglutinin (HA) to create a dSosHA fusion construct. dSosHA was transiently expressed in COS-1 cells and immunoprecipitated with anti-HA antibodies. When immune complexes were incubated with purified MAP-2 kinase and [γ-^{32}P]ATP, a phosphorylated band of 180 kDa was observed when analyzed by SDS-PAGE. This band was not present in immunoprecipitations from cells transfected with vector alone. No phosphorylation of the 180 kDa band was seen when immunoprecipitates were incubated with [γ-^{32}P]ATP in the absence of MAP-2 kinase. Two dimensional analysis of tryptic peptides from dSosHA phosphorylated by MAP kinase *in vitro* revealed two major phosphorylated species that were also found in dSosHA isolated from COS-1 cells labeled with $^{32}P_i$. These results are consistent with the hypothesis that a feedback loop exits wherein growth factor activated MAP kinases phosphorylate and regulate Sos proteins. Studies are being conducted to determine if MAP kinase phosphorylation affects the function of Sos. An initial study on Sos function was done to determine the oligomerization state of Sos. In order to do this, the carboxyl terminus of dSos was tagged with the human c-myc epitope to create dSosmyc. COS-1 cells were transfected with both dSosHA and dSosmyc. HA immunoprecipitates from these cells were analyzed on Western blots for the presence of dSosmyc. Under conditions of full serum stimulation, dSosmyc was not present in HA immunoprecipitates from COS-1 cells that were transfected with both constructions. This data is consistent with the idea that Sos is a monomer.

I 313 PHOSPHORYLATION OF THE RAS SUPPRESSOR RSP-1 IS REGULATED BY RAS IN SIGNAL TRANSDUCTION PATHWAYS

Mary Lou Cutler and Laura Masuelli
Laboratory of Tumor Immunology and Biology, NCI, Bethesda Md 20892

Rsp-1 cDNA was isolated using an expression cloning assay designed to recover cDNA capable of suppressing *K-ras* transformation (Cutler et al Mol.Cell Biol. 12:3750-3756). When expressed under the control of a heterologous promoter *rsp-1* suppresses transformation of NIH3T3 cells by *Ki-ras* and *Ha-ras* but not *v-src*. The amino terminal two thirds of Rsp-1 consist of a series of leucine based repeats homologous to the leucine repeats in yeast adenylyl cyclase, a region necessary for activation of adenylyl cyclase by RAS in *Saccerevisiae*. To determine if Rsp-1 represents a target for Ras we assayed for Rsp-1 phosphorylation in a signalling system dependent upon Ras. Nerve growth factor (NGF), epidermal growth factor (EGF), or TPA were added to ^{32}P labeled PC12 cells. Following addition of growth factor, lysates of labeled cells were immunoprecipitated with antisera directed against the COOH terminus of Rsp-1 and analyzed by SDS PAGE. Within 5 minutes phosphorylation of Rsp-1 was detected in response to NGF, EGF, and TPA. Rsp-1 phosphorylation could also be detected in EGF or TPA treated NIH3T3 cells with similar kinetics. In addition, a time and concentration dependent proteolytic cleavage of Rsp-1 was observed in response to growth factor addition. The GSRas1 and the RasDN6 PC12-derived cell lines containing inducible v-Ras or dominant negative (Asn17) Ras, respectively, were provided to us by Dr. Simon Halegoua. In these cell lines the phosphorylation of Rsp-1 in response to NGF and EGF was inhibited by the induction of Asn 17 Ras, and induction of v-Ras resulted in phosphorylation of Rsp-1. These results suggest that Rsp-1 functions "downstream" of Ras on signal transduction pathway(s) from extracellular growth factor receptors. *In vitro* binding of Rsp-1- glutathione transferase fusion proteins to Ha-Ras suggests that these two proteins have the capability to interact directly. Collectively, these results suggest that Rsp-1 may be a component of a Ras signalling complex in both extracellular receptor and TPA initiated signal transduction pathway(s).

I 314 ACTIVATION OF THE *RAS/cAMP* PATHWAY IN *SACCHAROMYCES CEREVISIAE* BY *CDC25*

Corey Davis[1], Sara Jones[2], and James Broach[1]. [1]Dept. of Molecular Biology, Princeton University, Princeton, NJ 08544. [2]Dept. of Pharmocological and Physiological Science, University of Chicago, Chicago, IL 60637.

In *Saccharomyces cerevisiae* the Ras proteins (Ras1p and Ras2p) play a central role in controlling cell growth, executing their function through a cycle of GTP/GDP exchange and GTP hydrolysis. The activation state of Ras is controlled by two classes of regulatory proteins. The gene products of *IRA1* or *IRA2* convert Ras to the inactive GDP-bound state by stimulating GTPase activity. The product of the *CDC25* gene catalyzes a GDP-to-GTP exchange to activate Ras.

To understand in detail the molecular mechanism by which Cdc25p activates Ras2p, we have identified, by mutation, domains of Ras2p that may interact with Cdc25p. We predicted that mutations in *RAS2* that diminish the interaction of Ras2p with Cdc25p will exhibit a Ras$^-$ phenotype. We also predicted that such mutations would be suppressed by the dominant intragenic point mutation, *RAS2*^{val19}, known to activate Ras2p by eliminating its dependence on Cdc25p. We expected that some of these mutations may be suppressed by overexpression of *CDC25* on a high copy yeast episomal plasmid. Preliminary genetic evidence suggests that three of these mutations reduce Ras2p function by diminishing its interaction with Cdc25p, and may define the domains of the Ras protein which physically interact with Cdc25p.

Although *RAS* is known to be intricately involved in nutrient responses, the only nutrient to which it reponds is glucose. The glucose signal for activation of the pathway is believed to be transmitted through Cdc25p, but a protein responsible for this transmission has yet to be identified. Problems with previous genetic screens have been identified, and we believe we have developed reliable screens which may uncover this elusive upstream activator. Preliminary evidence suggests that (under certain conditions) the gene for this factor causes an activated phenotype when expressed on a high copy yeast episomal plasmid.

I 315 A NOVEL DOMAIN THAT INTERACTS WITH ACTIVATED RAS, Susan D. Demo[1], Stanley Fields[3], Akira Kikuchi[1], Christopher Schneider[2], Yen-Wen Chen[1], Anthony J. Muslim[1], Franz Hofer[2], G. Steven Martin[2] and Lewis T. Williams[1], [1]University of California, San Francisco, 505 Parnassus Ave, Box 0130, San Francisco, CA 94143, [2]Department of Molecular and Cell Biology, 401 Barker Hall, University of California, Berkeley CA 94720 and [3]Department of Microbiology, S.U.N.Y. at Stonybrook, Stonybrook, NY, NY 11794

The product of the Ha-*ras* proto-oncogene is a member of a family of small GTP-binding proteins that also includes the products of the *ral, rac* and *rho* genes. A number of activating mutations in this protein have been found but little is known about the effectors of activated Ras. The cytoplasmic serine/threonine kinase Raf appears to be one candidate for a downstream effector of Ras.

Using the two-hybrid system to screen for proteins that bind to H-Ras, we identified partial cDNAs for two proteins that contain a novel domain that interacts with Ras: a previously uncharacterized mouse ras binding protein (mRBP) and the human homolog of the mouse or rat guanine nucleotide dissociation stimulator (GDS) for the Ral protein (hralGDS). A minimal domain of 94 amino acids that is 57% identical among these proteins and is C-terminal to the catalytic domain of ralGDS is sufficient to bind Ras. This domain does not bind the small GTP-binding proteins, Rac or Ral. The interaction of Ras and the 94 amino acid domain depends on the activation of Ras by GTP and is eliminated by mutations which disrupt Ras effector function. Using recombinant GST-Ras fusion protein and mRBP1, we showed in vitro association of these proteins depends on GTP-bound Ras. These findings suggest that ralGDS and mRBP maybe novel effectors of Ras.

I 316 **TRAFFICKING OF THE β_2-ADRENERGIC RECEPTOR FOLLOWING LIGAND-INDUCED INTERNALIZATION** Burton Dickey, Robert Moore, Nicholas Sadovnikoff, Simon Hoffenberg, Shaobin Liu, Kimon Angelides, and Brian Knoll, Departments of Pulmonary Medicine and Cell Biology, Baylor College of Medicine, Houston, TX 77030.

We are interested in studying the regulated trafficking of G-protein coupled receptors through the endosomal pathway, and in particular how these pathways are defined and regulated by Rab proteins. For these purposes, we are using a human embryonal kidney cell line which expresses 200,000 N-terminal epitope-tagged human β_2-adrenergic receptors (β_2AR) per cell. Following exposure to the agonist isoproterenol for 15 min, 60% of receptors become inaccessible to the hydrophilic ligand [^{3}H]CGP12177, but all receptors remain accessible to the hydrophobic ligand [^{3}H]DHA. After the removal of agonist, most of the sequestered β_2AR again becomes accessible to [^{3}H]CGP12177. Following exposure to isoproterenol for 180 min, 90% of receptor is inaccessible to [^{3}H]DHA, suggesting receptor degradation. Using laser confocal microscopy, we have observed the movement of receptors from a linear surface distribution into punctate vesicles within 5 min of agonist treatment, and the colocalization of β_2AR with Rab5 and with transferrin receptors (TfR). No apparent redistribution of Rab5 or TfR is observed after isoproterenol exposure. After removal of isoproterenol, punctate intracellular β_2AR returns to a linear surface distribution with a time course similar to that measured by binding studies. Following exposure to isoproterenol for 180 min, β_2AR increasingly colocalizes with mannose 6-phosphate receptors, and TfR-containing compartments which do not contain β_2AR become apparent. Dominant suppressor mutants of Rabs 4, 5 and 7 have been constructed and are being used to examine the effects of their expression on agonist-induced sequestration of receptors, on the recycling of receptors to the surface, and on receptor down-regulation.

I 317 PDGF-INDUCED, Ca^{2+}-PERMEABLE CHANNELS IN MESANGIAL CELLS: ACTIVATION INVOLVES A GTP-BINDING PROTEIN AND TYROSINE KINASE. Douglas C. Eaton, Hiroshi Matsunaga and Brian N. Ling, Emory Univ., Depts. of Med. & Physiol., Atlanta, GA.

Platelet-derived growth factor (PDGF) is a potent mesangial cell mitogen which also induces Ca^{2+} influx. Using cell-attached patch clamp technology on cultured rat glomerular mesangial cells, we have identified a very low conductance (0.7 pS, 110 mM Mn^{2+} as a charge carrier) Ca^{2+}-permeable channel which is activated by the addition of human recombinant PDGF-BB (50 ng/ml) to the pipette solution (JASN 3: 813, 1992). To further characterize this PDGF-induced channel, we used amphotericin-B "perforated" whole-cell patches. PDGF induced a small inward current (-16.1 $\pm$ 4.33 pA; n = 11 at a membrane potential of -70 mV). Using this value, single channel data, and a measured cell capacitance of 27.3 pF (n = 13), we calculated a channel density of 3000-4000 channels per mesangial cell or 4 X 10^7 channels/cm^2 suggesting a role for the channel in PDGF-mediated Ca^{2+} influx. We further investigated potential mechanisms for PDGF-induced channel activation. Application of 100 μM genistein, a tyrosine-kinase inhibitor, to the extracellular bath inhibited PDGF-induced channel activity (5 out of 5 cell-attached patches). In another set of experiments, PDGF-induced channel activity which had run-down with patch excision was restored by adding 200 μM GTPγS to the "cytoplasmic" bath (6 out of 8 inside-out patches). The inhibitory effect of genistein was abolished by GTPγS addition to the "cytoplasmic bath (3 out of 3 inside-out patches). In summary, PDGF-BB activates a very low conductance, Ca^{2+}-permeable channel in rat mesangial cell membranes only when applied close to the channel (within the patch pipette), but not when applied outside the pipette. This channel is only slightly voltage-dependent and would normally appear as a cation non-selective channel with a permeability to divalent cations $\geq$ univalent cations. The PDGF-induced channel provides a ligand-gated pathway for Ca^{2+} entry into mesangial cells which would not require membrane depolarization. PDGF likely functions through tyrosine-kinase-mediated phosphorylation and a GTP-binding protein.

I 318 EGF RECEPTOR EXPRESSION AND ACTIVITY IN CELLS TRANSFECTED WITH H-ras AND v-myc ONCOGENES, Tali Ehrlich[1], Gerald Princler[2], Mayumi Ono[2], Noah Isacov[1], Mark Smith[2], Hsiang-fu Kung[2], Michael Tainsky[3] Shraga Segal[1] Gordana Brkic[1] and Jacob Gopas[1], [1]Department of Microbiology and Immunology, Ben-Gurion University, Beer-Sheva, Israel, [2]Laboratory of Biochemical Physiology, Frederick Cancer Research and Development Center, P.O.Box B, Frederick 21702-1201, [3]Department of Tumor Biology, University of Texas, MD Anderson Hospital, Houston, TX 77030

EGF or EGF-like factors, deliver a mitogenic signal to the cell which is transduced with the participation of cellular protooncogenes such as ras. Derangement of protooncogene expression (i.e. oncogene activation) modifies the cellular response to growth factors. To investigate functional relationships between the EGF receptor (EGFR) and oncogenes, we have characterized the expression of the EGFR in H-ras, v-myc and (H-ras + v-myc) transfected Balb/3T3 cells.

H-ras cells show a marked decrease in EGF binding in comparison to parental cells. v-myc and (H-ras + v-myc) cells bind intermediate amounts. No such changes were observed in insulin, transferrin and PDGF binding assays. The mitogenic response to EGF was further examined. H-ras and (H-ras + v-myc) cells do not respond to EGF. By contrast, EGF stimulates DNA synthesis in parental cells and v-myc transfected cells. Western blot analysis of EGFR and EGFR Tyrosine-phosphorylation (P-Y) upon EGF treatment, showed very low levels of EGFR in H-ras and (H-ras + v-myc) cells, while parental and v-myc cells showed substantial amounts, which were P-Y after EGF treatment. Levels of RAF, PLC-γ-1 and GAP where comparable in all the cells. P-Y after EGF treatment of PLC-γ-1 was observed in parental and v-myc cells but not in H-ras or (H-ras + v-myc) cells. P-Y GAP was not detected in H-ras and (H-ras + v-myc) cells, while parental and v-myc cells expressed constitutively phosphorylated GAP which was unaffected by EGF treatment.

These results suggest that growth promoting signals by EGF where not transmited in H-ras cells due to virtual absence of EGFR molecules. Lack of a mitogenic response in (H-ras + v-myc) cells, and low EGFR amounts detected despite the capacity of the cells to bind EGF, may be explained by possible conformational changes of the receptor in these cells, undetected by the antibody used, and/or by a possible defect in the EGF intracellular signal transduction pathway, affected by the combined expression of H-ras and v-myc.

I 319 POST-TRANSLATIONAL REGULATION OF P56^{lck} BY ACTIVATORS OF PROTEIN KINASE C. OPPOSING EFFECS OF THE TUMOR PROMOTER, PMA, AND THE NON-TUMOR PROMOTER, BRYOSTATIN, Dalia Galron*, Amnon Altman# and Noah Isakov*, *Departement of Microbiology and Immunology, Ben Gurion University of the Negev, Beer Sheva 84105, Israel; #Division of Cell Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037 USA

The lymphocyte-specific protein tyrosine kinase, p56^{lck}, undergoes serine phosphorylation and SDS-PAGE mobility alteration after stimulation of T cells with anti-CD3 mAb or with the PKC activator PMA. The significance of these events to the enzyme's activity and their relevance to the cell proliferation process are yet unclear. In a previous study we have shown that Bryostatin (Bryo), another PKC activator, induced increased degredation of PKC that correlated with inhibition of PMA-induced human T cell proliferation. Since p56^{lck} serves as a potential downstream target for PKC we tested whether PMA and Bryo will differentially affect its activity in T cells. Bryo, similar to PMA, induced a size shift of p56^{lck} towards p60^{lck} form within 5 min of stimulation. The effect of PMA was prolonged and lasted over 24 h, while that of Bryo was transient (8 h) and correlated in its time kinetic with the Bryo-induced PKC degradation. PMA stimulation of cells that were pretreated 24 h with Bryo did not induce a size-shift of p56^{lck}, in accordance with the suppositions that the shift is regulated by PKC and that Bryo induces PKC degradation. The *in-vitro* enzymatic activity of LCK was independent from its molecular mass, as determind by SDS-PAGE, since p56^{lck} and p60^{lck} exhibited similar autophosphorylation and enolase phosphorylation activity. However, p60^{lck} from 1 h PMA or Bryo-treated cells, but not p60^{lck} from 24 h PMA-treated cells, was tyrosine phosphorylated *in-vivo*. Althogh phosphorylation and dephosphorylation of LCK is likely to determine its three dimensional structure and activation state, we assume that differential phosphorylation may determine also its interactions with distinct cellular substrates. Preliminary data support this assumption by showing that a tyrosine-phosphorylated polypeptide of a 36kDa co-precipitate with the CD4-p56^{lck} complex from long-term PMA-treated, but not from Bryo-treated, cells.

I 320 Protein kinase A antagonizes platelet-derived growth factor induced mitogen-activated protein kinase signaling in human arterial smooth muscle cells and NIH3T3 cells. Lee M. Graves*†, Karin E. Bornfeldt‡†, Elaine W. Raines‡, Brian C. Potts*, Susan G. Macdonald§, Russell Ross‡, Edwin G. Krebs*¶. Department of Pharmacology* and Department of Pathology‡, University of Washington, School of Medicine, Seattle, WA 98195 and Onyx Pharmaceuticals§, Richmond, CA 94806, USA.

Stimulation of aortic smooth muscle cells with platelet-derived growth factor (PDGF-BB) leads to the rapid activation of MAP kinase kinase (MAPKK) and MAP kinase (MAPK). Physiological and pharmacological compounds that elevate cAMP and activate protein kinase A (PKA) such as prostaglandin E-2, isoproterenol, cholera toxin and forskolin, were found to inhibit the PDGF-BB-induced activation of MAPKK and MAPK. Forskolin, but not the inactive analog 1,9-dideoxy forskolin, inhibited PDGF-BB-stimulated MAPKK and MAPK activation in a dose dependent manner. PKA antagonism of MAPK signaling was observed at all doses of PDGF-BB or PDGF-AA. PKA did not inhibit MAPKK and MAPK activity *in vitro*, however, MAPKK and MAPK from extracts of forskolin-treated cells could be activated normally with purified Raf-1 and MAPKK, respectively, *in vitro*, suggesting that PKA blocked signaling upstream of MAPKK. Neither PDGF-BB-stimulated tyrosine autophosphorylation of the PDGF receptor β-subunit nor inositol monophosphate accumulation were affected by increased PKA activity, suggesting that PKA inhibits events downstream of the PDGF receptor. In NIH3T3 cells, a similar inhibition of EGF or PDGF-stimulated MAP kinase signaling by PKA was observed. In these cells, the insulin stimulation of the p70 S6 kinase was also inhibited by forskolin pre-incubation. The results of the present study are an example of cross-talk between two important signaling systems activated by physiological stimuli, namely the protein kinase A pathway and the growth factor-activated MAPK cascade. Furthermore, these results show that a protein kinase pathway (p70 S6 kinase) not directly related to the MAP kinase cascade was also inhibited by PKA in cells.

I 321 DUAL REGULATION OF THE RAS EXCHANGE PROTEIN, VAV, BY DIGLYCERIDES AND TYROSINE KINASES. Erich Gulbins[1], K. Mark Coggeshall[1], Alfred Wittinghofer[2], Paul Burn[3], Shulamit Katzav[4] and Amnon Altman[1], Division of Cell Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037[1], Max Planck Institute for Molecular Physiology, Dortmund, Germany[2], Hoffmann-LaRoche Ltd, 4002 Basel, Switzerland[3], Lady Davis Institute, Montreal, Quebec H3T 1E2, Canada[4]

We recently identified Vav as a Ras-activating guanine nucleotide exchange factor (GEF) stimulated by antigen receptor-coupled protein tyrosine kinases (PTKs). Here, we examined the specificity of Vav towards several Ras-related proteins, determined the role of the cysteine-rich, PKC-homologous Vav domain, and addressed the physiological role of Vav and its interaction with Ras in intact cells. *In vitro*-translated purified Vav activated by PMA or by phosphorylation with recombinant p56^{lck} displayed GEF activity against Ras but not against recombinant RacI, RacII, Ral or RhoA proteins. PMA, diacylglycerol (DAG) or ceramide treatment of intact T cells, Vav immunoprecipitates or affinity-purified Vav generated by *in vitro* translation or COS-1 cell transfection stimulated its Ras exchange activity in the absence of detectable tyrosine phosphorylation. Stimulation was resistant to PTK and PKC inhibitors, but was blocked by calphostin, a PMA/DAG antagonist. *In vitro*-translated purified Vav lacking its cysteine-rich domain, or mutated at a single cysteine residue within this domain (C528A), was not stimulated by PMA, but was fully activated by p56^{lck}. The physiological significance of this alternative pathway is suggested by the finding that recombinant interleukin 1α, in contrast to T cell receptor agonists, stimulated Vav in intact T cells in a calphostin-sensitive, but PTK-independent manner, suggesting that diglyceride-mediated Vav activation may couple PTK-independent receptors which stimulate production of phospholipid-derived second messengers to Ras in hematopoietic cells. Stable expression of proto- or onco-Vav in NIH 3T3 cells led to a ~15-fold increase in basal or PMA-stimulated Ras exchange activity, respectively, in total cell lysates or Vav immunoprecipitates. Elevated GEF activity was paralleled in each case by a significant increase in the proportion of active, GTP-bound Ras. Furthermore, MAP kinases, known downstream intermediates in Ras-dependent signaling pathways, exhibited increased basal activity in onco-*vav*-transfected cells. These results demonstrate a physiologic interaction between Vav and its target, Ras, that leads to MAP kinase activation, and establish Vav as a specific and physiological Ras activator that is subject to dual, independent regulation by PTKs and lipid second messengers.

I 322 *Abstract Withdrawn*

I 323 BIOCHEMICAL CHARACTERIZATION OF Cdc25p, THE GUANINE NUCLEOTIDE EXCHANGE FACTOR FOR THE RAS PROTEINS OF *SACCHAROMYCES CEREVISIAE*. Steven A. Haney and James R. Broach, Department of Molecular Biology, Princeton University, Princeton, NJ 08544.

In *S. cerevisiae*, adenylate cyclase is controlled by Ras1p and Ras2p. Activation of these Ras proteins is in turn controlled by Cdc25p, which facilitates the exchange GDP bound to Ras for GTP. We have studied the Cdc25p-mediated nucleotide exchange reaction *in vitro*, using Ras2p and the catalytic fragment of Cdc25p that were expressed and purified as fusion proteins to glutathione-S-transferase. We have shown that the K_m for Ras•GDP is 175 nM, and the K_m for Ras•GTP is only two-fold higher. Using free GDP instead of free GTP in the exchange reaction raises the K_m from 25 μM to 68 μM, and lowers the k_{cat} from 28 min^{-} to 17 min^{-}. The effect of guanine nucleotides on k_{cat} suggests that the nucleotide association step is rate limiting. The K_m and k_{cat} values for nucleotide exchange by Cdc25p are more similar to those of GTPase stimulation by NF1-GAP, than by p120-GAP, for Ras•GTP. The results suggest that Cdc25p functions as a general guanine nucleotide exchange factor, rather than one that acts specifically on GDP-bound Ras2p. In related studies, we have characterized the effect of a dominant negative Ras2p mutant on Cdc25p-mediated nucleotide exchange. The dominant negative proteins examined, $Ras2p^{ala22}$ and $Ras2p^{gly19ala22}$, are potent inhibitors of Cdc25p activity, and have an affinity for Cdc25p that is over two orders magnitude stronger than wild type Ras2p, as determined by an interference assay.

I 324 ENHANCED BINDING OF TRANSCRIPTION FACTORS TO DNA IN THE PRESENCE OF DETERGENTS, Hamdy H. Hassanain, Sook Young Chon, and Sohan L. Gupta, Hipple Cancer Research Center, 4100 South Kettering Boulevard, Dayton, OH 45439

Studies reported earlier (Hassanain, *et al.* Anal Biochem 213:162-167, 1993) showed that the binding of several transcription factors (AP-1, SP1, GATA-1, ISGF3) to DNA probes was greatly enhanced (up to 20-fold) in the presence of neutral detergents such as CHAPS, CHAPSO, NP-40, as assayed in cell extracts. The sensitivity of the gel mobility shift assay for DNA-binding factors tested is greatly increased in the presence of neutral detergents. The binding specificity, however, is not compromised. Further studies have been carried out to determine the basis of CHAPS stimulated binding of AP-1 factor to DNA with nuclear extracts. Results indicate that enhanced binding of AP-1 to DNA in the presence of CHAPS is not due to an increased stability of AP-1-DNA complex. Scatchard analysis of binding results with increasing concentration of DNA probe indicated no significant change in binding affinity but a substantial increase in binding sites. Our results suggest that a bulk of AP-1 activity is sequestered in nuclear extracts and is released by CHAPS detergent. The results have practical implications to studies on DNA-binding factors.

I 325 BIOCHEMICAL AND FUNCTIONAL CHARACTERIZATION OF A RECOMBINANT GTP-BINDING PROTEIN, RAB5, AND TWO OF ITS MUTANTS, S. Hoffenberg, J. Sanford, S. Liu, M. Tuvin, M.A. Wessling-Resnick, B.J. Knoll, and B.F. Dickey, Baylor College of Medicine, Houston, TX; Harvard School of Public Health, Cambridge, MA.

The small GTPase Rab5 appears to regulate the fusion of early endosomes *in vivo* and *in vitro*, although studies have not been carried out using purified, recombinant proteins. Wild type and site specific mutant cDNAs were cloned into a bacterial expression vector, and the proteins were purified from *E. coli* lysates. The biochemical properties of the following Rab5 mutant proteins were compared to wild type protein: Q79L, which is the cognate of the Ras GTPase deficient mutant Q61L, and N133I, a guanine-nucleotide binding defective mutant. After transfer to nitrocellulose, WT protein bound [α-^{32}P]GTP with high affinity and hydrolyzed [γ-^{32}P]GTP; the Q79L mutant also bound GTP with high affinity but hydrolyzed the nucleotide to a much lesser extent; the N133I mutant displayed no apparent binding of nucleotides. GTPase activity measured in equilibrium conditions was limited by the GDP dissociation rate of the proteins (k_{off} = 0.014 and 0.004 min^{-1} for Q79L and WT, respectively). Therefore, the equilibrium GTPase rate of Rab5WT is 3 times that of RabQ79L, while the initial rate is 10 times greater. Dissociation rates for triphosphate nucleotides for Rab5WT and Rab5Q79L were about the same (GTPγS - 0.002 and 0.002, GTP - 0.004 and 0.004, respectively). In the presence of GDP, GTPγS or Gpp(CF_2)p, trypsin cleaved a fragment (~ 5,000 Da) from Rab5WT and Rab5Q79L; in the absence of nucleotides or in the absence of Mg^{2+}, proteins were further degraded. ATP did not protect either protein from degradation. Rab5N133I protein was protected from proteolysis by GDP (10^{-2} M) but not by GTPγS. With GDP no cleavage of the original protein could be detected by SDS-PAGE. Wild type Rab5 prenylated by reticulocyte lysate in the presence of mevalonate activated endosome fusion *in vitro*. Further endosome fusion studies are being done with Rab5 mutant proteins, and mutant proteins have been stably transfected into a $ß_2$AR-overexpressing cell line for *in vivo* study.

I 326 EXPRESSION CLONING OF A NOVEL ONCOPROTEIN, OST, THAT REGULATES ACTIVITIES OF RHO, RAC AND CDC42

Yoshihiro Horii, Kazushige Sakaguchi* and Toru Miki
*Laboratory of Cellular and Molecular Biology, National Cancer Institute; and *Metabolic Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases; National Institutes of Health, Bethesda, Maryland 20892.*

The Rho family small GTP-binding proteins including Rho, Rac and CDC42, function as molecular switches in signal transduction pathways for cytoskeletal organization. These proteins in their inactive GDP-bound form are converted to the active GTP-bound form by guanine nucleotide exchange factors, whereas the GTP-bound form is rendered inactive by GTP hydrolysis assisted by GTPase activating proteins (GAPs).

We constructed an expression cDNA library in λpCEV27 from a rat osteosarcoma cell line. The DNA was introduced into NIH/3T3 cells and several transformed foci were isolated. A plasmid containing a cDNA insert, designated *ost*, was rescued from one of the transformants. The Ost protein contains the dbl homology (DH) motif, which is found in regulators of the Rho family GTP-binding proteins. Ost can function as a guanine nucleotide exchange factor on RhoA and CDC42, and a GAP for Rac1. Moreover, Ost can specifically associate with GTP-bound Rac1. Therefore, Ost is implicated in a critical regulatory component which links the signal transduction pathways through Rac1, RhoA and CDC42.

I 327 GROWTH FACTOR STIMULATED PHOSPHORYLATION, DNA BINDING AND NUCLEAR TRANSLOCATION OF ATF-2

Stephen M. Jackson, Hany Abdel-Hafiz, Tere Marcell, James L. McManaman, Richard J. Sanderson and James P. Hoeffler. Division of Medical Oncology, Univ. of Colo. HSC, Denver, CO 80262

The cellular control mechanisms that regulate growth and differentiation are influenced by hormone- and growth factor-stimulated signal transduction pathways. These pathways converge at the level of the nucleus to influence the expression of genes which dictate cell phenotype. We have previously demonstrated that mitogen activated protein kinases (MAP-kinases) phosphorylate activating transcription factor-2 (ATF-2), a member of the CREB/ATF leucine zipper family. Phosphorylation of ATF-2 by MAP kinases stimulates the DNA binding activity of ATF-2. In the present study, we extend the previous results and examine the effects of growth factors on the activity of ATF-2. In vivo phosphorylation of ATF-2 occurs rapidly in response to epidermal growth factor, a known MAP kinase stimulator. This phosphorylation event correlates temporally with the translocation of ATF-2 into the nucleus. Furthermore, we demonstrate that the DNA binding activity of ATF-2 is stimulated in growth-factor stimulated cells. The stimulation of DNA-binding activity occurs with a similar temporal profile as the translocation into the nucleus. In addition, we provide evidence that the intracellular compartmentalization of ATF-2 varies during specific stages of the cell cycle. Interestingly, ATF-2 is predominantly nuclear in the stages of the cell cycle when MAP kinases are known to be active. These data suggest that ATF-2 nuclear localization and DNA binding activity can be regulated by MAP kinase pathways. Thus, we propose that ATF-2 provides a downstream target that acts as a pivotal effector of the serine/threonine protein kinases which are activated by growth factor receptor/tyrosine protein kinase pathways.

I 328 IMMOBILIZED RAS INTERACTION WITH RAF1 AND MEK-1 FROM CULTURED FIBROBLASTS IS INDEPENDENT OF CELL STIMULATION

Tomas Jelinek, Shonna A. Moodie, Paul Dent, Alan Wolfman, Thomas W. Sturgill, and Michael J. Weber, University of Virginia School of Medicine, Box 441, Charlottesville Va 22901

Recent studies have established that a direct interaction occurs between c-Ha-Ras and Raf-1, implicating Raf as a major effector of Ras function. It is at present unclear whether this association is responsible for and affected by the activation of Raf. We have examined the interactions of Raf-1 and MEK-1 from NIH 3T3 cells with immobilized Ras conjugated to silica. All the detectable Raf-1 in cell lysate was associated with Ras:GMPPNP, irrespective of serum stimulation. The association was sensitive to the addition of a synthetic peptide corresponding to the effector domain of Ras, but not to other peptides examined. In addition, MEK-1 was found to associate with immobilized Ras:GMPPNP, but was not quantitatively depleted from the lysate. Binding of MEK-1 was sensitive to the addition of Ras effector domain peptide, as was Raf, suggesting that binding of the two proteins requires the same Ras sequences. Analysis of supernatants from binding reactions indicated that depletion of Raf from the lysate precluded further MEK-1 binding, despite large amounts remaining in the supernatant. Further experiments indicated that MEK-1 is able to associate stably with Raf-1, suggesting that the two proteins associate with Ras as one complex whose formation is regulated solely by the presence of Ras:GTP *in vivo*.

I 329 FORSKOLIN INHIBITS A VOLTAGE-ACTIVATED POTASSIUM CURRENT IN NEUROBLASTOMA CELLS BY A cAMP-INDEPENDENT MECHANISM

Dearing W. Johns and Joyce K.W. Blandino, Cardiovascular Division, University of Virginia School of Medicine, Charlottesville, VA 22908

Forskolin (FSK) activates adenylate cyclase (AC) and has been used as a tool to evaluate the role of cAMP as a second messenger of hormonal influence on membrane currents and ion channel activity. The forskolin homolog, 1,9-dideoxyforskolin (ddFSK) does not activate AC nor cause a rise in cAMP. Mouse neuroblastoma (Neuro-2A) cells were differentiated in serum-free DMEM media for 48-72 hrs. Whole-cell outward potassium currents were elicited in response to 200ms depolarizing pulses to +90mV (10mV increments) from a holding potential of -80 mV. Application of 10^{-5}M FSK or ddFSK to the bath reduced the steady-state potassium current, I_K, within 5 minutes in paired experiments (mean ±SEM, pA/pF):

	Control	FSK	n	p
I_K	21.5±4.0	8.1±2.1	(7)	<0.001
	Control	ddFSK		
I_K	23.1±6.0	5.0±2.6	(6)	<0.01

Samples of media and cells incubated with 10^{-5}M FSK or ddFSK for 5 minutes were acetylated and acetyl-cAMP was determined by automated radioimmunoassay (n+3, mean ±SEM, pmoles/ml, *=p<0.05):

	media	cells
Control	2±0.3	20±3
FSK	22±12*	135±4*
Control	2±0.03	7±2
ddFSK	5±1	9±2

The reduction in steady-state I_K suggests an increase in the rate of inactivation. I_K was reduced by both FSK and ddFSK while only FSK increased cAMP concentration. Thus, forskolin inhibits the steady-state potassium current by a cAMP-independent mechanism.

I 330 CELL CYCLE DEPENDENT REGULATION OF KB-BINDING ACTIVITY CORRELATES WITH IKB PHOSPHORYLATION

Larry D. Kerr, Dept. of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-2363.

The ubiquitous transcription factor, NF-κB stimulates the expression of a number of genes involved in immune responses, acute phase reactions, and viral infections. The large array of growth factors, cytokines, and viral proteins which activate κB-binding activity suggests that the NF-κB family might play a pivotal role in the regulation of such cellular processes as proliferation, differentiation, and cell death. Studies using the pre-B cell line, 70Z/3 and the mature B cell line, WEHI231 indicate that κB-binding activity is regulated in a cell cycle dependent manner. Little or no detectable κB activity is observed during the period corresponding to G_1 while high levels of κB activity are detected in S phase cells. This is not due to an alteration in the mRNA or protein levels for the κB-binding factors or the inhibitor IκB, MAD3, as determined by northern and western blot analyses. Differences are observed in the pattern of phosphorylation of MAD3 at the G_1/S border which map to phosphorylated serine residues in the ankyrin repeat region of the IκB. Mutagenesis studies are underway to define the exact sites of phosphorylation. In addition, in vitro and in vivo studies detailing the inhibitory properties of the mutant IκB proteins will be presented. A mechanistic examination of the factors and signals which regulate NF-κB activity throughout cell cycle traverse will further our understanding of the events which effect not only normal growth and differentiation, but the aberrant processes associated with autoimmune loss of tolerance, chronic infection, and neoplastic transformation.

I 331 INHIBITION OF RAS-DEPENDENT CELL TRANSFORMATION BY INHIBITORS OF FARNESYL-PROTEIN TRANSFERASE, N. E. Kohl, N.J. Anthony, J. Davide, S.J. DeSolms, E. Giuliani, R.P. Gomez, S.L. Graham, S.D. Mosser, D.L. Pompliano, G.C. Prendergast, R.L. Smith, A. Oliff and J.B. Gibbs, Departments of Cancer Research and Medicinal Chemistry, Merck Research Laboratories, West Point, PA 19486.

The enzyme farnesyl-protein transferase (FPTase) catalyzes the post-translational addition of a farnesyl group to the cysteine residue located in a carboxyl-terminal CaaX tetrapeptide of a number of cellular proteins, including Ras. Since the Ras oncoprotein requires this modification for proper subcellular localization and cell-transforming activity, inhibitors of FPTase may be effective agents in the treatment of Ras-dependent human tumors. The two substrates of the farnesylation reaction, farnesyl diphosphate (FPP) and CaaX tetrapeptides, serve as models for the design of FPTase inhibitors. We have identified a CaaX tetrapeptide analog and three structural classes of FPP mimics which are all potent and specific inhibitors of FPTase in vitro. A prodrug of the CaaX analog, L-731,734, and one of the FPP mimics inhibited FPTase in cells. L-731,734 blocked the anchorage independent growth of *ras*-transformed Rat1 cells. Furthermore, compounds related to L-731,734 caused morphological reversion of the Rat1*ras* cells in monolayer culture. These studies demonstrate inhibition of *ras*-dependent cell transformation with synthetic organic inhibitors of FPTase.

I 332 **INTERACTION OF GTP-BOUND FORM OF HA-RAS WITH RAF-1,** Hiroshi Koide[1], Sheri Miraglia[2], Takaya Satoh[1], Martin McMahon[2], Yoshito Kaziro[1], [1]Tokyo Institute of Technology, Yokohama, Japan, and [2]DNAX Research Institute, Palo Alto, CA

Ras is involved in signaling pathway for cellular growth and differentiation, and transmits signals from tyrosine kinases to MAP kinases. Recently, Raf-1, a serine/threonine kinase, has been identified as a possible direct downstream target of Ras. Several groups have demonstrated the interaction of Ras and Raf using immobilized Ras•GTP and yeast two-hybrid system. We have observed the association between Raf-1 and Ha-Ras by immunoprecipitation method. Recombinant Ha-Ras either in GTP- or GDP-bound form was incubated with cell lysates, which was then immunoprecipitated with an anti-Ras antibody (LA069). Raf-1 was co-precipitated with the GTP-bound form of Ras, but not with the GDP-bound form. The association was occurred in the lysates of several cultured cell lines including NIH3T3, PC12, Ba/F3 and Jurkat, as well as in the crude extracts of rat brain. The results indicate that the association between Ras and Raf is a general mechanism in signal transduction through Ras. In addition, by using Ha-Ras mutants, we found that an "effector" region of Ha-Ras is involved in this association. We are now examining whether the binding of Ras•GTP to Raf is sufficient or the other molecule(s) is required for the Raf kinase activation.

I 333 IDENTIFICATION OF NEW GENES INVOLVED IN Ras-MEDIATED SIGNAL TRANSDUCTION IN *C. elegans*.
Kerry Kornfeld, Greg Beitel, and Bob Horvitz, HHMI, Dept. of Biology, MIT, Cambridge, MA 02139

During vulval development in the nematode *C. elegans*, the anchor cell signals adjacent Pn.p cells to adopt vulval fates. The response to this signal requires a pathway mediated by the *let-23* receptor tyrosine kinase, *let-60 ras* and *lin-45 raf*. An activating, codon 13 mutation in *let-60 ras* causes extra Pn.p cells to adopt vulval fates resulting in a multivulva (Muv) phenotype. To identify genes involved in vulval development, particularly genes that act downstream of Ras, we screened for mutations that suppress this Muv phenotype. Forty three independent mutations were identified that define 22 complementation groups; we named these *suppressor of activated ras* (*sar*).

Three complementation groups appear to be required for all signal transduction because mutants display the same spectrum of phenotypes as *let-23* and *let-60 ras* mutants. One group corresponds to the previously identified *lin-45 raf* gene while *sar-1* (7 alleles) and *sar-2* (6 alleles) have not been previously identified. We are trying to clone these two genes by using their genetic map positions and the well characterized *C. elegans* physical map. Mutations in two complementation groups, *lin-39* and *sar-3* (1 allele), affect the differentiation of Pn.p precursor cells and thus block vulval development prior to Ras activation. Mutations in two complementation groups, *lin-25* and *sar-4* (4 alleles) affect the execution of vulval differentiation. Mutations in 15 complementation groups did not independently affect vulval development. In collaboration with M. Lackner and S. Kim (Stanford), we have shown that one of these complementation groups, containing the single allele *n2521*, corresponds to a *C. elegans* homolog of MAP kinase: *mpk-1*. Thus a *C. elegans* MAP kinase homolog acts downstream of Ras and plays an important role in Ras signaling.

I 334 HAKs: A MAP KINASE SUBFAMILY ACTIVATED BY HEAT STRESS, John M. Kyriakis, Papia Banerjee, Eleni Nikolakaki†, Bernd J. Pulverer†, Mir F. Ahmad, James R. Woodgett† and Joseph Avruch, Diabetes Research Laboratory, Massachusetts General Hospital East, Charlestown, MA 02129 and †The Ontario Cancer Institute, Princess Margaret Hospital, Toronto, Ontario, Canada M4X 1K9

We report here the molecular structures of a family of four mammalian 54-kDa protein Ser/Thr kinases which are related to the mitogen-activated protein kinases (MAPKs) (≈50% identity to *erks 1* and *2*) as well as *FUS3/KSS1* from *S. cerevesiae* (≈45% identity). This kinase subfamily includes the p54 MAPK originally purified from the livers of cycloheximide-treated rats. The four isoforms, αI (p54 MAPK from rat liver), αII, β and γ share >90% sequence identity with the αI and αII isoforms arising as mRNA splice variants. In order to understand the regulation of these kinases we raised a polyclonal antiserum against the β-isoform. This serum specifically immunoprecipitates the 54-kDa kinases from cell extracts. Using GST-c-Jun as a substrate, we observe that the 54-kDa kinases are not activated by mitogens, phorbol esters or DNA damage (UV, or nitrogen mustard), but are potently activated by heat shock and protein synthesis inhibitors (cycloheximide). By contrast, the p42/p44 MAPKs (Erks 2 and 1) are strongly activated by mitogen and only poorly activated by heat shock. Accordingly, we have named these 54-kDa kinases heat stress-activated kinases (HAKs). Our results indicate that heat shock signaling may utilize components similar to, yet distinct from those recruited by the more familiar mitogen signaling pathways.

I 335 KINASE SIGNALLING PATHWAYS ACTIVATED IN *X. laevis* OOCYTES BY *ras* ONCOGENES AND PHOSPHOLIPASES

J.C. Lacal, A. Carnero, F. Dolfi and B. Jiménez

Instituto de Investigaciones Biomèdicas, CSIC, Madrid, Spain

Induction of oocyte maturation by hormones and the oncogenic *ras*-p21 protein has been shown to correlate with a cascade of phosphorylations of the Ser/Thr family of kinases. On the other hand, signal transduction induced by generation of second messengers from membrane phospholipids is considered a major regulatory mechanism in the control of cell proliferation. In the *Xenopus laevis* oocytes model, microinjection of the three most relevant types of phospholipases (PLA_2, PLC and PLD) is capable of inducing oocyte maturation through generation of their known second messengers. A number of critical Ser/Thr kinases conserved in evolution are involved in this process. We have investigated the activation of cdc2 kinase, MAP kinase and S6 kinase II by phospholipases A_2, C and D and have correlated their mechanisms of activation with that of *ras*-p21 proteins in the model system of *X. laevis* oocytes. We show that all phospholipases analyzed were able to activate MPF, but only PLD was able to activate MAP kinase and S6 kinase II. We also report that PLD-induced but not PLC- or PLA_2-induced activation of cell cycle kinases correlated with the *ras*-p21 signaling pathway. All these results indicate that *ras*-p21 functions upstream of PLD and its biological activity may be mediated by generation of phosphatidic acid in *Xenopus laevis* oocytes. Finally, the use of specific inhibitors of phospholipases and protein kinase C made it possible to identify several alternative and independent signalling pathways that induce oocyte maturation. *ras*-p21 proteins can function independently of phospholipase C or protein kinase C activities. *ras*-p21, as well as phospholipase D, induced the rapid production of phosphatidic acid and phospholipase A2 activation in a $p42^{MAPK}$-dependent manner. While inhibition of PLA2 by quinacrine does not interfere with *ras*- or PLD-induced GVBD, phosphatidic acid itself induced similar signalling that *ras* or PLD reinforcing the notion that *ras*-p21 is functionally linked to PLD.

I 336 **A *C. elegans* homolog of MAP kinase, *mpk-1*, is involved in vulval development.**

Mark Lackner[1], Kerry Kornfeld[2], H. Robert Horvitz[2], and Stuart K. Kim[1]. [1]Department of Developmental Biology, Stanford University Medical Center, Stanford, CA 94305. [2] Department of Biology, M.I.T., Cambridge, MA 02139.

During development of the *C. elegans* hermaphrodite, three of six developmentally equivalent cells P3.p-P8.p are signaled by the gonadal anchor cell to adopt vulval fates. The response to this signal is mediated by a tyrosine kinase signal transduction pathway that has been remarkably well conserved during metazoan evolution. Because mitogen-activated protein (MAP) kinases are activated by tyrosine kinase pathways in vertebrate cells, we reasoned that *C. elegans* MAP kinase homologs might play a role in vulval induction; two *C. elegans* MAP kinase genes, *mpk-1* and *mpk-2*, were cloned using a degenerate PCR approach. Independently, we sought to identify genes involved in vulval induction by screening for mutations that suppress the vulval defects caused by a constitutively active *let-60 ras* protein. These studies have converged on the same gene, as one of these suppressors is an allele of *mpk-1*. We have also demonstrated by mosaic analysis that *mpk-1* acts cell-autonomously in the vulval precursor cells. These results extend previous biochemical studies by showing that MAP kinase activity is required for *ras*-mediated cell signaling *in vivo*.

I 337 THE ROLE OF THE STE20 PROTEIN KINASE AND STE5 IN YEAST PHEROMONE SIGNAL TRANSDUCTION,

Ekkehard Leberer, Daniel Dignard, Doreen Harcus, Malcolm Whiteway and David Y. Thomas, Biotechnology Research Institute, National Research Council of Canada, 6100 Avenue Royalmount, Montreal, Quebec H4P 2R2, Canada

The mating pheromone response in the yeast *Saccharomyces cerevisiae* is a genetically tractable model system to study the molecular mechanism of a signal transduction pathway regulated by a heterotrimeric G protein. The β and γ subunits of the G protein have been shown to be responsible for transmitting the pheromone signal to downstream signalling components which include yeast homologues of mitogen activated protein (MAP) kinases. We have identified in a genetic screen a novel gene, designated *STE20*, encoding a putative protein Ser/Thr kinase which is a potential target for the G protein β and γ subunits (Leberer *et al.*, 1992, *EMBO J.* **11**, 4815-4824). By searching for high gene-dosage suppressors of a Ste20 protein kinase mutant, we have been able to isolate the *STE5* gene whose product is essential for pheromone signal transduction and has structural and functional similarity to Far1, a pheromone-responsive inhibitor of G_1 cyclins. Genetic interactions between *STE20*, *STE5* and other "sterile" genes indicate that the Ste20 protein kinase and the Ste5 protein are involved in the regulation of a protein kinase cascade that links a G protein coupled receptor to yeast homologues of MAP kinases.

I 338 KNOCK-OUT OF THE PDGF GENES IN MICE
Per Levéen, Christer Betsholtz, and Milos Pekny, Department of Pathology, University of Uppsala, Sweden

Platelet-derived growth factor is a family of homo- and heterodimeric ligands built up by two polypeptide chains, A and B. The PDGFs interact with two different receptor molecules, *pdgfra* and *pdgfrb*. The A-chain appears to have affinity only for *pdgfra* whereas the B-chain can bind to both receptors. The current model of receptor activation involves receptor dimerization followed by autophosphorylation in *trans*, which permits subsequent interaction with exogenous substrates for the receptor protein tyrosine kinase. The PDGF chains as well as the PDGF receptors are expressed during embryonic development. For example, the appositional expression of PDGFA and *pdgfra* suggests a role for this ligand-receptor pair in the formation of mesenchymal structures. In support of this, the mouse *patch* mutation, a deletion which encompasses the *pdgfra*-gene (and possibly additional genes), leads in its homozygous state to severe connective tissue defects and to death before birth.

In order to increase our understanding of the *in vivo* roles of the PDGFs, we have knocked out the PDGF genes in embryonic stem cells with the aim of generating specific PDGF-isoform deficiencies in mice. Aspects of the PDGF B-chain-negative phenotype will be presented at the meeting.

I 339 CHARACTERIZATION OF RAS- AND TPA-STIMULATED c-Jun N-TERMINAL KINASES, Anning Lin and Michael Karin Department of Pharmacology, University of California, San Diego, La Jolla, CA 92093

c-Jun is a phosphoprotein. The phosphorylation of two N-terminal activation domain sites, Ser63 and Ser73, is stimulated by a Ha-Ras dependent signal transduction pathway and correlated with the trans-activation activity of c-Jun. Two novel Serine/Threonine protein kinases, p55 and p46, have been purified 10,000-fold using FPLC system from TPA/ionomycin-stimulated Jurkat cells. These two c-Jun N-terminal kinases have no detectable ERK antigenic activity, as demonstrated by Western blot using anti-ERKs antibody. Both p55 and p46 were autosphorylated in in-gel kinase assay, and they also significantly phosphorylated GST-c-Jun (1-223) which was precasted in the SDS-gel. Two-dimentional phosphopeptide mapping revealed that the phosphorylation sites of c-Jun p55 and p46 were Ser63 and Ser73. The molecular cloning of these two novel c-Jun kinases is in progress.

I 340 POTENTIATION OF EGF RECEPTOR TRANSFORMING ACTIVITY BY C-SRC.
Ming-Chei Maa, Tzeng-Horng Leu, and Sarah J. Parsons. Dept. of Microbiology, University of Virginia, Charlottesville, VA 22908.

Parallels between levels of cytosolic protein tyrosine kinase (PTK) activity and stages of maligancy in human breast tumors have been reported. At least 70% of the PTK activity originates from the c-src proto-oncogene product (Cancer Res. 52: 4773-4778, 1992). In addition, overexpression of two closely related receptor tyrosine kinases, EGF receptor (EGFR) or $p185^{neu}$, has been correlated with poor prognosis of breast tumors. Here, we present evidence that $pp60^{c-src}$ can potentiate both EGF-dependent mitogenesis and EGFR transforming activity in murine fibroblasts overexpressing both kinases as compared to cells overexpressing either c-src or EGFR alone. This potentiation correlates not only with the formation of a heterocomplex between $pp60^{c-src}$ and activated EGFR, but also with the appearance of a novel EGF-dependent tyrosyl phosphorylation on the receptor, suggesting that the receptor is phosphorylated by $pp60^{c-src}$. That this phosphorylation may lead to an enhancement of receptor enzymatic activity is evidenced by the increased level and duration of phospholipase Cγ (PLCγ) tyrosyl phosphorylation after EGF stimulation. Based on these findings, we suggest that $pp60^{c-src}$ potentiates EGFR oncogenic capacities by binding to and phosphorylating ligand-bound receptors, thereby increasing their ability to phosphorylate critical downstream substrates. In addition, cell lines overexpressing both EGFR and c-src may be useful models for future breast tumor studies.

I 341 IN VITRO RAS-RAF BINDING AND ITS EFFECTS ON THE ACTIVATION OF RAF-1. Susan Macdonald, Tony Evans, Emilio Porfiri, Janice Williams and Frank McCormick. Onyx Pharmaceuticals, Richmond, CA 94806

Many studies have shown that Raf-1 signals downstream of Ras and is dependent on Ras for its activation. The precise mechanism by which Ras contributes to the activation of Raf, however, is not known. A clue has been provided by the recent evidence from several laboratories showing that Raf-1 binds directly to Ras, but the effect of this binding on the activation of Raf-1 has not yet been assessed. We have closely examined the *in vitro* binding of Raf-1 to ras using several forms of Ras and Raf. Using epitope-tagged forms of Ras and Raf-1 expressed in Sf9 cells, we have found that Raf-1 binds with higher affinity to both the GTP-bound and prenylated forms of H-Ras and K-Ras. We have also been able to examine the relative binding affinities of activated Raf-1 and kinase-dead Raf-1 for Ras. Furthermore, we have studied the effects of Ras-Raf-1 binding on the activity of Raf-1 by using the physiological Raf-1 substrate, MEK, in *in vitro* kinase assays. We have found that the simple binding of Ras to Raf-1 does not activate Raf-1, although previously activated Raf-1 bound to Ras is able to phosphorylate MEK quite well. Nor is Raf-1 bound to Ras able to be activated by protein kinase C, although Raf-1 is phosphorylated by protein kinase C in this context. We are currently screening for cellular factors that are able to activate Raf-1-bound to Ras. Our results regarding the characterization of the binding of full length Raf-1 to Ras and the *in vitro* activation of Raf-1 will be presented.

I 342 THE RAS-GAP SH3 DOMAIN SUPPRESSES TRANSFORMATION BY MUSCARINIC RECEPTOR M5,
Ian G. Macara*, Alexander Sorisky*, Mark Brann and Ray Mattingly*, Departments of Pathology* and Psychiatry, University of Vermont, Burlington, VT 05405-0068

The N-terminal half of Ras-GAP contains an SH2-SH3-SH2 motif. SH3 domains are known to bind to proline-rich sequences, but their functions are not clear, and there are very few biological assays for SH3 domain-dependent processes. We have established a focus assay using NIH 3T3 cells that allows an analysis of Ras-GAP SH3 domain structure/function relationships. The human muscarinic receptor subtypes 1,3 and 5, which couple through Gq to phospholipase C, are capable of agonist-dependent transformation of NIH 3T3 cells. Co-transfection of the GAP N-terminal domain suppresses focus formation in this assay. We have generated various fragments of the N-terminal domain and found that the isolated SH3 domain, but not the SH2 domains, are capable of suppressing focus formation. This effect is specific to muscarinic receptors, as no inhibition of focus formation is observed using v-Ras or c-trkA as the transforming agents. Point mutations in the SH3 domain of NGAP abolish the inhibitory activity. The inhibitory effect is not a result of suppression of muscarinic receptor expression. Agonist stimulation of muscarinic receptors in 3T3 cells activates endogenous c-Ras. Experiments to determine whether the inhibitory effect of the GAP SH3 domain is upstream or downstream of Ras will be described.

I 343 IMMUNOCHEMICAL IDENTIFICATION OF DIFFERENT PRODUCTS OF CDC25^{Mm} IN MAMMALIAN TISSUES,
E.Martegani, R.Zippel, C.Ferrari, N.Gnesutta, V.Carrera, L.Alberghina, E.Sturani. Dipartimento di Fisiologia e Biochimica Generali, Università di Milano, 20133 Milano, Italy.
The CDC25^{Mm} gene encodes for Ras exchange factor(s) originally identified by functional complementation of the *Saccharomyces cerevisiae cdc25*-1 mutant. Four different full lenght cDNAs (called CDC25^{Mm} type I, II, III and IV) have been isolated from mouse brain and Northern blot analysis indicates that it is mainly expressed in brain, although CDC25^{Mm} product were identified in PC12 cells and in NIH3T3 fibroblasts. In order to study the expression of the products of CDC25^{Mm} gene in different mouse tissues we have prepared two polyclonal antibodies directed towards two different region of the CDC25^{Mm} proteins comprised in the last 472 aminoacids. In most of the tested tissues (heart, skeletal muscle, kidney, liver and testis) we were unable to clearly identify CDC25^{Mm} products while two different products were observed in brain. A large protein of approx 140 kDa, likely corresponding to the protein encoded by form IV cDNA, was present in adult mouse brain, while a smaller protein was predomimant in brain of embryos and in postnatal mouses, suggesting that the expression of CDC25^{Mm} is developmentally regulated. In addition a poorly expressed 120 kDa protein was identified in NIH3T3 fibroblasts. The expression of CDC25^{Mm} gene was monitored also with RT-PCR experiments, and we found positive signals in most tissues when the sequence corresponding to the C-terminal region was amplified, while a positive signal with amino terminal region of form IV cDNA was detected only in brain.

I 344 MULTIPLE FUNCTIONAL DOMAINS OF THE p21RAS ACTIVATOR SON OF SEVENLESS.
L.S. McCollam*, L. Bonfini+, L.M. Kozma*, K. Baltensperger*, A.D. Cherniack*, U. Banerjee+, and M.P. Czech*. *Program in Molecular Medicine and Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, MA, 01605. +Department of Biology, University of California at Los Angeles, Los Angeles, CA, 90024.

Previous work indicates that insulin activates Ras by increasing the exchange of its GDP for GTP (1). Genetic studies of *Drosophilia melanogaster* have identified a gene encoding son of sevenless (dSos), the putative activator of Ras (2). Cotransfection of cDNAs encoding dSos and human H-Ras increased the amount of GTP bound Ras in ^{32}P-labeled COS-1 cells relative to cells transfected with H-Ras alone (3). In addition, dSos associates with insulin receptor substrate-1 (IRS-1) complexes, containing PI-3 Kinase, in an insulin dependent manner (3). One interaction of dSos with IRS-1 signaling complexes occurs via the association of the SH3 domain of Grb2 with the proline rich C-terminal domain of dSos.

In the present studies, dSos deletion mutants which lack the C-terminal 480 amino acids including the proline rich domain (NcatdSos), or the domain amino terminal to the putative catalytic region (CatCdSos) were tagged with the hemagglutinin (HA) epitope at the C-terminus for the purpose of determining whether these mutants could associate with the IRS-1 signaling complex and its associated PI-3 kinase activity. COS-1 cells were cotransfected with each of the dsosHA deletion mutant cDNAs and the human insulin receptor (hIR) or a catalytically inactive mutant of the receptor (hIRA/K). Cell lysates were immunoprecipitated with anti-IRS-1 or anti-HA antiserum and analyzed for the presence of dSos, IRS-1, and PI-3 kinase. The results revealed that both NcatdSos and CatCdSos are able to associate with IRS-1 signaling complexes consisting of PI-3 kinase in response to native hIR. Significantly, as expected, NcatdSos did not associate with Grb2 under the conditions of these experiments. NcatdSos but not CatCdSos was able to cause increased Ras· GTP loading when transfected into COS-1 cells.

These data indicate that the C-terminal Grb-2 binding region of dSos is not necessary for insulin receptor-dependent association of dSos with IRS-1 signaling complexes. These studies reveal the potential importance of the N-terminal domain of dSos in its function.

1. Medema, R.H., *et al.*, Mol. Cell. Biol. 13, 155 (1993).
2. Bonfini, L., *et al.*, Science 255, 603 (1992).
3. Baltensperger, K., *et al.*, Science 260, 1950 (1993).

I 345 CALCIUM BLOCKS EGF- INDUCED P21RAS- AND ERK- ACTIVATION IN PRIMARY HUMAN KERATINOCYTES.
J.P. Medema, C. Backendorf*, M.Sark*, J. L. Bos. Laboratory for Physiological Chemistry, Utrecht University, 3521GG Utrecht and *University of Leiden, Leiden, The Netherlands.

Primary human keratinocytes proliferate in an epidermal growth factor (EGF)-dependent manner as long as the extracellular calcium concentration is below 1mM. Raising [Ca^{2+}] results in the onset of differentiation. Most studies have focussed on the late events after EGF- and Ca^{2+}-addition, but little is known about the early signal transduction. We therefore focussed our attention on the immediate effects of EGF and Ca^{2+} on these cells.

We found that EGF potently activated p21^{ras} as measured by an increase in the level of p21^{ras}GTP. This increase reached a maximum after 5 minutes and was back to basal levels after 30 minutes. EGF addition also resulted in ERK activation which had similar kinetics as p21^{ras} activation except that it reached its peak activity after 10 minutes. This ERK activation was dependent on p21^{ras}, since ras(asn17) expression completely blocked the EGF-induced activation. Ca^{2+} addition on the other hand did not activate p21^{ras} nor ERK, suggesting that the p21^{ras}/ERK signalling cascade is connected to the proliferation pathway.

Addition of both EGF and Ca^{2+} to keratinocytes has been shown to result in the onset of differentiation indicating that Ca^{2+} can overrule the EGF-induced proliferation. The dominant effect of Ca^{2+} can also be shown at the molecular level since addition of Ca^{2+} inhibited the EGF-induced activation of p21ras and ERK. This inhibition was transient and overruled by high concentrations of EGF. Ca^{2+} addition however did not affect the EGF receptor autophosphorylation indicating that Ca^{2+} blocks in between the receptor and p21^{ras}.

I 346 TERMINAL DIFFERENTIATION AND SENESCENCE IN THE MELANOCYTE: INABILITY TO PHOSPHORYLATE THE MITOGEN-ACTIVATED PROTEIN KINASE 2 (erk2) SELECTIVELY DEFINES THE TWO PHENOTYPES, EE Medrano, F Yang, R Boissy, K Matsumoto, JJ Nordlund and H-Y Park*, Department of Dermatology, University of Cincinnati College of Medicine, Cincinnati, OH and *Department of Dermatology, Boston University School of Medicine, Boston, MA.

Human melanocytes, whether derived from adult or neonatal skin, proliferate well in a medium supplemented with phorbol esters before they undergo senescence. In this communication we delineate the conditions for terminal differentiation which is induced by cholera toxin in the melanocyte isolated from human adult epidermis. We show that cells became round, flat and enlarged. These changes were accompanied by a 5-6 fold increase in cAMP levels and in melanin content. Mitogen-activated kinases (MAPKs, also called extracellular signal-regulated kinases) are rapidly activated in response to ligand binding to receptors. This family of kinases appears to integrate multiple intracellular signals transmitted by various second messengers. By using Western blot analysis and an anti-phosphotyrosine antibody we found that phorbol esters induced in proliferating neonatal and adult melanocytes a rapid tyrosine phosphorylation of the extracellular signal-regulated kinase ERK2. In contrast, senescent and terminally differentiated cells were unable to phosphorylate tyrosine residues of the ERK2 gene product in spite of presenting normal amounts of ERK2 protein. These results suggest that both senescent and terminally differentiated melanocytes share a common block in a critical pathway, which would prevent the continuation of the signal transduction cascade initiated by PMA activation of PKC.

I 347 ACTIVATIONS OF PHOSPHOLIPASE D AND MAP KINASE ARE INDEPENDENT SIGNALING EVENTS, Kathryn E. Meier, Cynthia D. Bradshaw, Krishna M. Ella, Katrina C. Gause, and Linda G. Jones, Department of Cell and Molecular Pharmacology, Medical University of South Carolina, Charleston, South Carolina 29425

Phospholipase D (PLD) and mitogen-activated protein kinases (MAPK) are rapidly activated in a variety of cells in response to growth factors and other mitogenic agonists. Phosphatidic acid, product of the PLD reaction, is proposed to play a role in mitogenic signal transduction. This study examined whether activation of MAPK was dependent on activation of PLD. In the A7r5 vascular smooth muscle cell line, both PLD and MAPK are activated with similar time courses in response to phorbol ester (PMA) or vasopressin (VP); VP is a mitogenic agonist for these cells. Both enzymes are activated within five minutes after addition of agonist. However, the effects of VP on both PLD and MAPK are more transient than those of PMA. MAPK activity can be activated in response to a thromboxane A_2 receptor agonist in A7r5 cells; this agonist has no effect on PLD activity. When A7r5 cells are incubated with exogenous bacterial PLD, phosphatidic acid increases to a level similar to that seen in response to VP or PMA. However, bacterial PLD does not induce activation of MAPK, does not inhibit activation of MAPK in response to VP, and is not mitogenic in A7r5 cells. In the wild-type EL4 thymoma cell line, MAPK is activated in response to PMA. Phosphatidic acid levels are not increased by PMA in intact EL4 cells. PLD activity is low or non-existent in this cell line, as measured by an *in vitro* assay in broken cell preparations. Expression of PLD activity was found to be variable between different T cell lines. Addition of bacterial PLD to EL4 cells produces a massive increase in phosphatidic acid levels. However, this treatment does not increase MAPK activity in EL4 cells. These results indicate that, in two different types of mammalian cells, activations of MAPK and PLD are concurrent but independent events.

I 348 YEAST RAS2 PROTEINS CONTAINING A C-TERMINAL BASIC DOMAIN DO NOT REQUIRE PRENYLATION FOR FUNCTION, D.A. Mitchell, T.K. Marshall and R.J. Deschenes Department of Biochemistry, University of Iowa, Iowa City, IA 52242

Saccharomyces cerevisiae possess two Ras homologs at least one of which is required for viability. Ras proteins are made as soluble precursors which undergo a series of posttranslational modifications that localizes the protein to the cytoplasmic surface of the plasma membrane. The processing events include farnesylation, proteolysis, methylesterification and palmitoylation and are mediated through the terminal four amino acid residues of the protein known as the CaaX box. Mutations which prevent farnesylation abolish Ras activity. Although palmitoylation and methylation are not essential, they may increase the affinity of Ras for the membrane. In an attempt to understand the requirements for Ras localization, we mutagenized the Ras2p CaaX box and have identified mutants which do not contain a CaaX box, but still possess Ras activity. These mutants contain serine for cysteine substitutions followed by a basic amino acid extension of the sequence IIKLIKRK (Ras2(CC-ext), Ras2(SC-ext), Ras2(CS-ext), and Ras2(SS-ext)). The extension mutants, when expressed from a low copy number plasmid, are sufficient to support Ras-dependent growth in a strain in which the chromosomal copies of Ras have been deleted. In these strains, Ras2(CC-ext)p and Ras2(CS-ext)p have activities similar to wild-type Ras2p, whereas Ras2(SC-ext)p and Ras2(SS-ext)p have lower activities. In addition, strains utilizing Ras2(SS-ext)p are temperature sensitive for growth at 37° C. Removal of the basic residues from the extension mutants completely abolishes their activity. The extension mutant proteins are not prenylated. However, metabolic labeling with tritiated palmitic acid revealed that Ras2(CC-ext)p, Ras2(CS-ext)p, and Ras2(SC-ext)p are covalently modified by palmitate. The level of palmitoylation was dependent on the sequence of the C-terminus suggesting that palmitoylation of cysteine residues may be governed by amino acid sequence context. We conclude that several mechanisms exist which allow Ras2p to functionally associate with the plasma membrane. (Supported by a grant from the National Cancer Institute, CA 50211)

I 349 CONTROL OF PROTEIN SYNTHESIS DURING T-CELL ACTIVATION AND ITS INHIBITION BY IMMUNOSUPPRESSANT AGENTS, Suzanne Miyamoto[1], Gavin Welsh[2], Chris Proud[2], and Brian Safer[1]. Molecular Hematology Branch, NHLBI, Bethesda, MD., 20892[1] and Dept. of Biochemistry, University of Bristol, Bristo. BS8, UK[2].

T cell activation can be initiated in quiescent G_0 T lymphocytes by mitogenic agents, such as PHA, anti-CD3, ionomycin and the phorbol ester, PMA. These agents triggers a cascade of events that ultimately results in the acquisition of immunologically competent T cell function and cellular proliferation. Protein synthesis is a critical component of T cell activation. It is required for the expression of early intermediate genes, i.e. IL-2, γ-IFN and IL-2R and also for DNA synthesis. Overall, the rate of protein synthesis increases 10 fold during the first 24 hours. The mechanisms leading to this increase is not well understood. Gene expression of three translation initiation factors eIF-2α, eIF2β and eIF-4α increases rapidly, 50 fold, within the first twelve hours, but only a 2-4 fold increase in each protein is observed at 24 hours. Phosphorylation of eIF-4α increases, but phosphorylation of eIF-2α does not. Increases in the activity of the guanine nucleotide exchange factor, eIF-2B has also been observed. Protein synthesis can be paritally inhibited in G_0 T cells after activation with ionomycin and PMA by immunosuppressive agents, cyclosporin, FK506 and rapamycin. Cyclosporin and FK506 have been shown to block early events (G_0-G1), therefore inhibiting the induction of the early genes, IL-2 and IL-2R. Rapamycin, however seems to inhibit later events of the activation process, before the onset of proliferation. Our interest is to study and identify the role and nature of this inhibition and determine how these events affect the control of protein synthesis. Post-translational events of certain translations initiation factors, i.e. phosphorylation of eIF-2α and eIF-4α have been identified as potentially having a regulatory role in protein synthesis. Increased phosporylation of eIF-4α has been correlated with increased translational activity in other tissues. The activity of the guanine nucleotide exchange factor of eIF-2, eIF-2B, also has been postulated to have a regulatory role in protein synthesis. We are currently exploring the role of these factors in regulating protein synthesis during T cell activation, and whether these activities are directly or indirectly inhibited by the actions of the immunosuppressants.

I 350 DIFFERENCE IN THE INTERACTION AMONG PKA, PKC, AND RAS SYSTEMS IS A POSSIBLE MECHANISM FOR THE SUBTYPE-SPECIFIC REGULATION OF LYMPHOKINE EXPRESSION IN T HELPER LYMPHOCYTES, Yoshiyuki Naito, Hideharu Endo, Robert L. Coffman, Ken-ichi Arai and Naoko Arai, DNAX Research Institute, Palo Alto, CA 94304-1104, and Institute of Medical Science, University of Tokyo, Tokyo 108, Japan.

T helper (Th) lymphocytes are categorized into at least two subtypes, Th1 and Th2, according to their lymphokine production patterns. Upon T cell receptor (TCR)-mediated stimulation, Th1 produces interleukin (IL)-2 and interferon (IFN)-γ, and Th2 produces IL-4, IL-5, IL-6, and IL-10. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-3 are produced by both Th1 and Th2. To investigate differential regulation mechanisms of lymphokine gene induction, we compared signal transduction pathways between Th1 and Th2, measuring lymphokine production as a readout under various stimulation conditions. Lymphokines were classified into three groups in terms of signal requirements, regardless of Th subtypes. GM-CSF and IL-2 required activation of both PKC- and calcium-mediated pathways, whereas production of IFN-γ, IL-3, and IL-4 was partially induced by calcium ionophore alone. IL-5, IL-6, and IL-10 were partially produced by PMA alone, and their production was relatively resistant to cyclosporin A. However, production of other lymphokines, which requires activation of the calcium-mediated pathway, was sensitive to cyclosporin A. The difference in signal requirements was also confirmed by transfection experiments using constitutively active PKC and constitutively active calcineurin. Of interest, transfection of an activated form of Ras (v-Ha-Ras) alone induced IL-5, IL-6, and IL-10 production. PGE_2 discriminated between Th1 and Th2 in TCR-mediated activation, that is, production of lymphokines was suppressed by PGE_2 in Th1 cells, but not in Th2 cells. Secretion of some Th2 lymphokines was stimulated instead. PMA-induced lymphokine production was affected in a similar manner, while calcium ionophore-induced lymphokine production was not affected by PGE_2. Both the stimulatory and inhibitory effects of PGE_2 were mimicked by dBcAMP and by transfection with PKA catalytic subunit. These results suggest that the cAMP-PKA system modulates the PKC-mediated pathway rather than the calcineurin-mediated pathway. Differential modulation of the PKC-mediated pathway through the cAMP-PKA system may give some clue for the differential regulation of lymphokine gene expression in the two subtypes of Th cells.

I 351 INVESTIGATION OF EFFECTOR DOMAINS OF RHO AND RAC USING CHIMERIC PROTEINS

Catherine Nobes, Dagmar Diekmann and Alan Hall, CRC Signal Transduction and Oncogene Group, MRC Laboratory for Molecular Cell Biology, University College London, Gower St., London WC1E 6BT, UK.

The small GTP-binding proteins Rho and Rac are closely related members of a subgroup of the large family of Ras-like proteins. Rho and Rac play a part in signalling pathways that link growth factor receptor activation to the regulation of actin assembly in cells. Quiescent Swiss 3T3 fibroblasts have little specialised F-actin but micro-injection of activated Rho protein induces stress fibre formation and new formation of focal contacts [1], whilst activated Rac affects actin organisation at the plasma membrane and resultant membrane ruffling [2]. To investigate the regions of each of these proteins that regulate their distinct biological activities we have created chimeric Rho/Rac proteins and looked at actin organisation after micro-injection into fibroblasts of these novel recombinant proteins. Our results so far indicate that the effector domain involved in stimulating stress fibre formation for Rho and ruffling for Rac is not, like ras, at amino acids 23-46 but instead is situated carboxy-terminal to amino acid 74. In addition we have begun investigating the regulation of RhoGDI by various growth factors that stimulate actin changes in cells. RhoGDI forms a complex in the cytoplasm with Rho and Rac and regulation of this complex formation may be key to the regulation of actin assembly in the cell.

1. Ridley AJ and Hall A. (1992) Cell 70, 389-399.
2. Ridley AJ, Paterson HF, Johnston CL, Diekmann D and Hall A. (1992) Cell 70, 401-410.

I 352 MOLECULAR ANALYSIS OF HUMAN PROTEIN FARNESYLTRANSFERASE AND PROTEIN GERANYL-GERANYLTRANSFERASE TYPE I: ENZYMES THAT MODIFY SIGNAL TRANSDUCTION PROTEINS POSSESSING A C-TERMINAL CAAX MOTIF, C. A. Omer, R. E. Diehl, A. M. Kral, M. D. Schaber, S. D. Mosser, D. L. Pompliano, K. S. Koblan, S. Graham, J.B. Gibbs and N.E. Kohl. Merck Research Laboratories, West Point, PA 19486

We are studying post-translational prenylation of signal transduction proteins, including the small GTP-binding proteins of the Ras-superfamily, that have a C-terminal CaaX sequence. Proteins in which the X residue is methionine, serine or glutamine (including all forms of Ras) are farnesylated (C15) by protein farnesyltransferase (FTase), while proteins in which the X residue is leucine (includes Rap1a, Rap1b, RhoA, RhoC) are geranylgeranylated (C20) by protein geranylgeranyltransferase type I (GGTase-I). Prenylation of these proteins is required for proper intracellular localization and function. Farnesylation is essential for cellular transformation by oncogenic forms of Ras. We have cloned and expressed human FTase and GGTase-I. These two heterodimeric enzymes share an identical subunit (α) while possessing a similar, but distinct, β subunit. Thus the specificity of the enzymes for both protein and isoprenoid substrates appears to reside in the corresponding β subunit. Enzymological, biochemical and site-directed mutagenesis analysis of cloned human FTase and GGTase-I have enabled us to characterize how the two distinct subunits of these enzymes interact with their substrates. Using recombinant human FTase we have discovered, by synthetic chemistry, potent inhibitors of the enzyme such as α-hydroxyfarnesyl phosphonic acid and a CaaX analog. Natural product screening has led to the identification of chaetomellic acid and zaragozic acids as FTase inhibitors. Each of these compounds is a significantly more potent inhibitor of FTase than of GGTase-I.

I 353 A MUTANT INSULIN RECEPTOR INDUCES SHC/GRB2 COMPLEX FORMATION AND MEDIATES RAS·GTP FORMATION WITHOUT TYR-PHOSPHORYLATION OF IRS-1, D.M. Ouwens[1], G.C.M. van der Zon[1], G.J. Pronk[2], J.L. Bos[2], and J.A. Maassen[1], [1]Department of Medical Biochemistry, Section Protein Synthesis and Hormone Regulation, University of Leiden, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands, [2]Laboratory for Physiological Chemistry, University of Utrecht, Vondellaan 24A, 3521 GG Utrecht, The Netherlands

Evidence is emerging that insulin receptors mediate Ras·GTP formation at least in part by Tyr-phosphorylation of IRS-1 and subsequent association to GRB2/SOS, whereas activated EGF-receptors interact directly with GRB2/SOS or indirectly via Shc. We investigated the contribution of the major Tyr-autophosphorylation sites, Y^{1158}, Y^{1162} and Y^{1163}, of the insulin receptor to the Tyr-phosphorylation of IRS-1 and Shc, and the formation of Ras·GTP. For that, we constructed CHO cell lines, overexpressing mutant insulin receptors in which the major Tyr-autophosphorylation sites were stepwise replaced by phenylalanines. It was found that insulin stimulates phosphorylation of IRS-1 and Shc on Tyr, and formation of IRS-1/GRB2 and Shc/GRB2 protein complexes, together with an increase in Ras·GTP, in cell lines expressing wild type or mutant $F^{1158}Y^{1162,1163}$ receptors. Cell lines expressing mutant $Y^{1158}F^{1162,1163}$ showed insulin-induced Tyr-phosphorylation of Shc, Shc/GRB2 complex formation and Ras·GTP formation, in absence of Tyr-phosphorylation of IRS-1 and formation of IRS-1/GRB2 complexes. The mutant insulin receptors $F^{1158,1162}Y^{1163}$ and $F^{1158,1162,1163}$ were inactive in inducing any of these responses. We conclude that phosphorylation of Y^{1158} and Y^{1162} of the insulin receptor is linked to distinct post-receptor processes, and that conversion of Ras·GDP to Ras·GTP can occur in absence of Tyr-phosphorylation of IRS-1, suggesting an involvement of Shc/GRB2 complex formation in insulin-induced Ras·GTP formation.

I 354 PHOSPHORYLATION AND ACTIVATION OF MAP KINASE AND RSK DURING PLATELET ACTIVATION AND AGGREGATION. *Jackie Papkoff, #Rey-Huei Chen, #John Blenis and *John Forsman. *Sugen, Inc., 515 Galveston Dr., Redwood City, Ca., 94063 and #Dept. Cell Biology, Harvard Medical School, Boston, Mass., 02115.

Human platelets provide an excellent model system for the study of tyrosine phosphorylation during signal transduction and cell adhesion. Platelets exhibit rapid phosphorylation on tyrosine of many proteins upon agonist induced activation and aggregation. We have sought to identify the kinases as well as the tyrosine phosphorylated substrates that participate in thrombin induced signal transduction and platelet aggregation. In this study we have identified two forms of MAP kinase (MAPK), p42 and p44, in platelets. The data demonstrate that p42 MAPK, but very little if any p44 MAPK, becomes phosphorylated on serine, threonine and tyrosine during platelet activation. Immune complex kinase assays, gel renaturation assays and a direct assay for MAPK activity in platelet extracts all support the conclusion that p42 MAPK, but not p44 MAPK, shows increased kinase activity during platelet activation. We have also identified p90 RSK, a previously characterized substrate for MAPK, in platelets. p90 RSK is phosphorylated on serine in resting platelets and the phosphorylation on serine is enhanced upon thrombin induced platelet activation. Immune complex kinase assays demonstrate that the activity of p90 RSK is markedly increased during platelet activation. Finally, we show that the increased phosphorylation and activity of both p42 MAPK and p90 RSK does not require integrin mediated platelet aggregation.

I 355 **INHIBITION OF THE S6 KINASE ACTIVATION PATHWAY.** Claudia Petritsch, Helga Edelmann and Lisa M. Ballou, Institute of Molecular Pathology, Vienna, Austria.

$p70^{S6K}$ is a mitogen-stimulated enzyme which phosphorylates the S6 protein of 40S ribosomal subunits on multiple sites. This phosphorylation is associated with an increase in protein synthesis and passage of cells through G_1. $p70^{S6K}$ is activated *in vivo* by phosphorylation of serine/threonine residues and can be inactivated *in vitro* by treatment with phosphatase 1 or 2A.

We identified 3 potent inhibitors of $p70^{S6K}$ activation: theophylline, SQ20006 and forskolin. Theophylline arrested cycling fibroblasts in G_1 and blocked the activation of $p70^{S6K}$ by various stimuli. Inhibition of the enzyme was most likely due to a block in its phosphorylation, as indicated by immunoblotting. Theophylline inhibited the activation of $p70^{S6K}$ reversibly and recovery from the inhibition did not require *de novo* protein synthesis. These results suggest that theophylline interferes with a kinase or activates a phosphatase upstream of $p70^{S6K}$.

Theophylline and SQ20006 are nonselective phosphodiesterase inhibitors that increase intracellular levels of cyclic nucleotides and switch on cyclic nucleotide-regulated pathways. The easiest explanation for the inhibition of $p70^{S6K}$ by theophylline is that the S6 kinase pathway is downregulated by cyclic nucleotides. However, we were able to rule out the possibility that cAMP or cAMP-dependent protein kinase are involved in the inhibition of $p70^{S6K}$ by theophylline. Currently we are investigating the role of the cGMP-dependent pathway in activation of $p70^{S6K}$.

I 356 **$p85^{s6k}$ IS TARGETED TO THE NUCLEUS AND REQUIRED FOR G_1 PROGRESSION**, Reinhard C., Fernandez* A., Lamb* N. and George Thomas, Friedrich Miescher Institut 4002 Basel, Switzerland; CNRS-INSERM 340033 Montpellier, France.

Increased 40S ribosomal protein S6 phosphorylation is argued to be a prerequisite for the activation of protein synthesis during G_1 progression. The kinase responsible has been identified as a Mr 70,000 protein, termed $p70^{s6k}$ (1), which is activated by phosphorylation of Ser/Thr Pro motifs. cDNA cloning revealed a second isoform termed $p85^{s6k}$, which is expressed from the same gene and is identical to $p70^{s6k}$ except for a 23 residue NH_2-terminal extention,which contains a nuclear localization signal (2,3). Immunofluorescence studies employing specific $p85^{s6k}$ antibodies show that endogenous and over expressed $p85^{s6k}$ localize to the nucleus. In addition the 23 amino acid extention is sufficient to target a heterologous fusion protein to this cell compartement. Anti-$p85^{s6k}$ inhibitory antibodies block G_1 progression when microinjected into the nucleus, but not the cytoplasm, of REF52 cells. Coinjection of anti-$p85^{s6k}$ IgG with active $p70^{s6k}$, lacking the antibody epitope, rescues the block of G_1 progression, demonstrating an essential functional role of the $p85^{s6k}$ in mitogenesis.

1. Kozma et al., (1990), PNAS 87, 7365-7369
2. Banerjee et al., (1990), PNAS 87, 8550-8554
3. Reinhard et al., (1992), PNAS 89, 4052-4056

I 357 RESOLUTION OF PHOSPHORYLATED AND NON-PHOSPHORYLATED EIF-4E AND DEMONSTRATION OF INCREASED CAP AFFINITY OF THE PHOSPHORYLATED FORM Robert E. Rhoads, Waldemar B. Minich, M. Luysa Balasta, and Dixie Goss, Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, Shreveport, LA 71130 and Department of Chemistry, Hunter College, New York, NY 10021

Eukaryotic initiation factor 4E (eIF-4E) plays a central role in the recognition of the 7-methylguanosine cap structure of mRNA and the formation of initiation complexes in protein synthesis. Previous studies have indicated that only eIF-4E which is phosphorylated at Ser-53 is active in protein synthesis. Stimulation of cells with mitogens leads to a simultaneous increase in protein synthesis and eIF-4E phosphorylation. Also, alteration of the phosphorylation site to Ala-53 prevents the transfer of eIF-4E to the 48S initiation complex. Finally, overexpression of eIF-4E (but not the Ala-53 variant) in cultured mammalian cells causes loss of growth control. Several studies have shown a direct involvement of the *ras* signal transduction pathway in the stimulation of protein synthesis via eIF-4E phosphorylation. Despite these findings, the biochemical basis for the observed differences between phosphorylated and nonphosphorylated eIF-4E is unknown. To address this directly, we have developed a chromatographic procedure for the separation of phosphorylated and nonphosphorylated forms of eIF-4E from rabbit reticulocytes. Using the resultant purified forms, we have studied the protein's interaction with various cap structures and have found a greater affinity using the phosphorylated form. The equilibrium binding constants for the interaction of phosphorylated eIF-4E with m^7GTP, m^7GpppG and globin mRNA were $20.0 \pm 0.1 \times 10^5\ M^{-1}$, $16.4 \pm 0.1 \times 10^5\ M^{-1}$, and $31.0 \pm 0.1 \times 10^5\ M^{-1}$, respectively. By contrast, the equilibrium binding constants for nonphosphorylated eIF-4E with m^7GTP, m^7GpppG and globin mRNA were $5.32 \pm 0.4 \times 10^5\ M^{-1}$, $4.52 \pm 0.4 \times 10^5\ M^{-1}$, and $10.0 \pm 0.12 \times 10^5\ M^{-1}$, respectively. This may account for the observed greater activity of phosphorylated eIF-4E. Supported by NIH Grant GM20818 and NSF Grants GER-9023681 and MCB-9303661.

I 358 IDENTIFICATION OF ESSENTIAL COMPONENTS OF PROTEIN KINASE C-MEDIATED INTERLEUKIN 2 GENE TRANSCRIPTION, Alison F. Richardson and Julianne J. Sando. Department of Pharmacology, University of Virginia, Health Sciences Center, Charlottesville, VA 22908.

Protein kinase C (PKC) activity is elevated in response to various hormones, growth factors and neurotransmitters, and leads to the coordinated transcription of a particular set of genes. The mechanisms through which PKC acts remain unclear and to further delineate these pathways PKC-mediated interleukin 2 (IL2) gene transcription was studied in phorbol ester-sensitive EL4 cells. Comparisons were made with a phorbol ester-resistant EL4 cell line, defective in this process. These studies have revealed increased tyrosine phosphorylation of an 85 KDa (p85) protein within 30 seconds of phorbol ester stimulation of sensitive EL4 cells. After 5 min, *lck* and *raf*-1 exhibited mobility shifts in SDS-PAGE and after 4 hours the presence of the transcription factor Fra (Fos-related antigen) was induced in nuclear extracts. In contrast, tyrosine phosphorylation of p85 protein was not detected in the resistant cell line; *lck* and *raf*-1 have deficient mobility shifts and the transcription factor Fra was not induced. Thus, a temporal series of events have been identified after phorbol ester stimulation and all of these are defective in resistant EL4 cells. Efforts are currently aimed at identifying p85 and determining if it has an essential upstream role for PKC-mediated IL2 gene transcription. Preliminary studies have suggested p85 may represent phosphatidylinositol 3-kinase. The nature and sites of p85 phosphorylation are also being pursued. [supported by NIH grant # DK40031].

I 359 CALCIUM REGULATION OF MAP KINASE KINASE, Laura B. Rosen, David D. Ginty, Michael J. Weber*, and Michael E. Greenberg, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, and *Department of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA, 22908

Membrane depolarization of neurons leads to calcium influx through voltage-sensitive calcium channels, which plays a critical role in transmitting signals in the nervous system. One of the downstream signaling events that occurs in response to calcium influx is stimulation of the mitogen-activated protein (MAP) kinase. MAP kinase is activated by a variety of signals that lead to cell growth and differentiation, but its function and mechanisms of activation in neurons are unknown. We have investigated the mechanism by which MAP kinase is activated in response to calcium influx in the pheochromocytoma cell line PC12. Calcium influx induced an activity that could directly phosphorylate and activate MAP kinase *in vitro* and that was chromatographically identical to the MAP kinase kinase (MEK) activity induced by nerve growth factor (NGF). To characterize this activity, we generated a polyclonal antibody to the N-terminus of mouse MEK1. This antibody recognized a major band of ~45 kD on Western blots that cofractionated with the peaks of MAP kinase kinase activity induced by calcium and NGF. *In vivo* phosphotryptic mapping demonstrated that calcium and NGF induced MEK phosphorylation on serine and threonine residues in the same set of tryptic peptides, suggesting that the same or functionally similar upstream kinases activate MEK in response to both stimuli. Since calcium and NGF lead to different long-term responses in PC12 cells, we examined whether their kinetics of MEK activation might be different. Using the anti-MEK antibody in an immune-complex kinase assay, we found that calcium induced MEK activity more transiently than did NGF, and these kinetics paralleled those of MAP kinase activation by the two stimuli. This result suggests that differential regulation of MEK activity is responsible for the different kinetics of MAP kinase activation. Our results identify MEK as a mediator of calcium signaling in PC12 cells and suggest that MEK may be important for calcium-dependent signaling processes in the nervous system. The different kinetics of MEK and MAP kinase activation in response to calcium and the neurotrophin NGF suggest a means by which diverse neuronal signals may use similar signaling intermediates that could ultimately program different outcomes.

I 360 UV-INDUCED C-FOS/C-JUN EXPRESSION INVOLVES GROWTH FACTOR RECEPTOR DEPENDENT SIGNALLING, Christoph Sachsenmaier, Adriana Radler-Pohl, Peter Herrlich and Hans J. Rahmsdorf, Inst.f.Genetik, Kernforschungszentrum Karlsruhe, P.O. Box 3640, 76021 Karlsruhe, Germany

UV, phorbol ester tumor promotors (e.g. TPA) and growth factors share common parts of a signal cascade, involving Raf- and MAP-kinases, that leads to the rapid posttranslational activation of transcription factors (e.g. Jun, Fos, SRF). We investigated the UV specific branch of the signal pathway leading to enhanced c-fos/c-jun gene expression and compared it with phorbol ester- and growth factor induced signalling.

'Cross-refractoriness' experiments were performed in order to identify agent-specific parts of the signal pathways. Pretreatment of HeLa cells with interleukin 1α (IL-1α), epidermal growth factor (EGF) or basic fibroblast growth factor (bFGF) leads to partial refractoriness to a subsequent stimulation of c-fos and c-jun expression by UV, while phorbol esters or the respective growth factors not used for pretreatment are still fully effective. Prestimulation with IL-1α, EGF and bFGF together leads to almost complete inhibition of UV-induced gene expression. This suggests that these growth factors and UV activate the same components, possibly the growth factor receptors, that are subsequently specifically downregulated. Supporting evidence for the involvement of growth factor receptors in UV signalling comes from the finding that suramin, a broad inhibitor of receptor-ligand interactions, completely blocks UV- and growth factor-, but not phorbol ester-induced c-fos/c-jun expression. Transient transfection of a dominant inhibitory H-ras mutant (asn-17) strongly interferes with UV induced expression of AP-1 dependent reporter constructs, while not interfering with phorbol ester induced expression. Additionally, in HeLa cells, UV, IL-1α and EGF cause hyper-phosphorylation of c-Jun that differs from phorbol ester induced phosphorylation.

Our results suggest, that UV signalling includes activation of growth factor receptors, initiating a signalling cascade via Ras, Raf and MAP kinase. Phorbol esters feed into this pathway at the level of Raf kinase. Hyperphosphorylation of Jun by UV and growth factors in HeLa cells may be due to the activation of additional kinases or to the differential activation of Raf kinase by phorbol esters and UV or growth factors.

I 361 THE HEPATITIS B VIRUS TRANSACTIVATOR MHBs^{T167} IS AN ER LOCALIZED PROTEIN THAT STIMULATES NF-*κ*B VIA REACTIVE OXYGEN INTERMEDIATES, Volker Schlüter[1], Markus Meyer[1], Patrick A. Baeuerle[2], Peter H. Hofschneider[1], and Wolfgang H. Caselmann[1,3], [1] Dept. of Virus Research, Max-Planck-Institut für Biochemie, [2] Laboratory for Molecular Biology, Genzentrum, Martinsried, and [3] Dept. of Medicine II, Klinikum Großhadern, University of Munich, Germany

As previously shown that C-terminal truncation of the hepatitis B virus middle surface proteins (MHBs) generates a novel transactivator termed MHBs^t. To further investigate the transactivatory potential a MHBs^t species we compare the transactivator protein MHBs^{t167} comprising only the N-terminal 167 amino acids of MHBs to the inactive full-length MHBs protein using the vaccinia virus/T7 polymerase expression system. Western blot analysis of HeLa cells infected with the recombinant viruses vT7S832 and vT7S486 expressing full length MHBs and C-terminally truncated MHBs^{t167}, respectively, indicates the expression of the exspected N-glycosylated forms. Immunoprecipitation of ^{35}S-methionine labeled MHBs and MHBs^{t167} revealed a similar stability of both proteins. For both proteins we could show a localization inside the endoplasmatic reticulum (ER) by double immunofluorescense staining. Cellular fractionation and western blot analysis of acetone precipitated supernatants of cells infected with vT7S832 or vT7S486 indicated that unlike MHBs, MHBs^{t167} is retained inside the secretory pathway.

Using different CAT reporter constructs we could demonstrate that MHBs^{t167} stimulates the NF-*κ*B dependent gene expression and causes nuclear appearence of NF-*κ*B in gel retardation assays. To investigate the signal transduction pathway of NF-*κ*B activation by the ER localized MHBs^{t167}, we tried to block the NF-*κ*B gene induction by the protein kinase C inhibitor H7 and by antioxidants. While the H7 treatment does not significantly influence the NF-*κ*B dependent gene activation, the antioxidants pyrrolidine dithiocarbamate (PDTC) and N-acetyl-L-cysteine (NAC) suppressed the MHBs^{t167} induced activation of NF-*κ*B. These data suggest that MHBs^{t167} induced stimulation of NF-*κ*B is mediated via reactive oxygen intermediates (ROI). Since ROIs can function as both tumor initiators and promotors, it is conceivable that the induction of oxidative stress by MHBs^{t167} may contribute to liver carcinogenesis.

I 362 RESIDUES CRUCIAL FOR RAS INTERACTION WITH GUANINE NUCLEOTIDE EXCHANGERS

Marisa Segal*, Berthe M. Willumsen# and Alexander Levitzki*, *Department of Biological Chemistry, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel and #Institute of Microbiology, University of Copenhagen, Øster Farimagsgade 2A, DK 1353 Copenhagen, Denmark.

We have developed a combined genetic-biochemical approach to characterize the interaction between the *Saccharomyces cerevisiae* Cdc25 protein, a Ras GDP-GTP exchanger, and mammalian Harvey-*ras* ($p21^{H\text{-}ras}$). We have shown that the *S. cerevisiae* Cdc25 protein and other related exchangers interact with $p21^{H\text{-}ras}$ expressed in yeast by promoting GDP-GTP exchange. Thus, the yeast system provides a convenient setup for studying in detail the interaction between various GDP-GTP exchangers of Ras proteins and mammalian $p21^{H\text{-}ras}$. We have devised a genetic screen for Ras mutants defective in catalyzed (Cdc25-dependent) exchange to identify residues in $p21^{ras}$ that are crucial for the interaction with the guanine nucleotide exchanger. We found that mutations within codon positions 97-105 impaired the ability of Ras to undergo catalyzed exchange without affecting its other biochemical functions including: the ability to interact with the effector, responsiveness to GAP and binding of guanine nucleotides. Detailed point mutation analysis will be presented.

I 363 THYMIC CARCINOMA IS DEVELOPED BY SV40 T ANTIGEN TRANSGENE, Jeong-Sun Seo, Department of Biochemistry, College of Medicine, Seoul National University, 28 Yeon-Geon Dong, Chong-Ro Gu, Seoul, 110-799, KOREA

SV40 T antigen has been used to produce tumors in transgenic mice by regulating its expression with a variety of transcriptional elements. A model of thymus cancer was produced in transgenic mice harboring the SV40 early region including viral own promoter and enhancer regions. Two independent lines of transgenic mice(line 227 and 248) were established in which nearly every affected animal succumbed with thymic carcinoma and kidney pathology when 5-7 months old. In two other lines (line 125 and 127) harboring the same DNA construct, some transgenic mice developed brain or thymic tumors, but their frequencies were much lower. Histological analysis of tumor tissue showed poorly differentiated carcinoma of thymus epithelial cells with aberrent mitotic figures. Immunohistochemical staining with monoclonal antibodies against SV40 T antigen showed that T antigen was expressed in the nucei of tumor cells. All mice examined also showed dysplastic renal tubules or kidney tumors. Metastatic foci of thymic cancer were observed at lung, liver, bone marrow and spleen. Brinster *et al.* previously produced transgenic mice harboring SV40 T antigen gene with its own regulatory region. They reported that most of the affected mice had developed choroid plexus tumor and that thymus hypertrophy and kidney pathology were observed in some mice. Our results showed that thymic tumor was the dominant finding in all mice of line 227 and 248 which developed thymic carcinoma without brain tumor. These results indicate that SV40 T antigen have different action or expression pattern in our transgenic mice. Cell lines were established from thymic tumor tissues. Immunoprecipitation of cell lysates with monoclonal antiboies showed that SV40 T antigen interacted with wild type p53 in our thymic cell line.

I 364 THE ROLE OF *CDC42Hs* IN THE CONTROL OF MAMMALIAN CELL POLARITY, Lisa Stowers, Debbie Yelon, Leslie Berg, and John Chant, Department of Biochemistry and Molecular Biology, Harvard University, Cambridge, MA 02138

In the budding yeast, *Saccharomyces cerevisiae*, the low molecular weight GTP-binding protein *CDC42Sc* has been implicated in the control of cell polarity. Temperature sensitive mutants of *CDC42* show enlarged, multinucleated cells that are incapable of establishing an axis of polarity. Remarkably, its human homologue maintains 80% identity across its amino acid sequence. We are investigating the function of this highly conserved protein in polarized mammalian cells. By sequence similarity to the CDC42Sc product we have created dominant interfering alleles of the protein which have been transfected into a T cell line. T cells are an effective model for polarity since both their T cell receptors and cytoskeleton rapidly and dramatically re-orient towards antigen as the T cell contacts the antigen presenting cell. We are continuing to characterize *CDC42* and its role in cell polarity establishment.

I 365 DEVELOPMENTAL EXPRESSION OF A RAS-RELATED PROTEIN IN PLASMODIUM FALCIPARUM, Chiang Syin and Neil Goldman, Laboratory of Parasitic Biology and Biochemistry, Office of Vaccine Research and Review, Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892

Malaria parasites, primarily an intracellular organism, have a complex life cycle in which they propagate through various morphological stages in vertebrate host and mosquito vector. The parasite is capable of expressing new sets of proteins during growth and stage differentiation. Although the secretory pathway in Plasmodium falciparum is presumably essential for the parasite's proliferation and interaction with the host, the components and the mechanism involved have received little attention until recently. We have several cDNA clones encoding a ras-related protein from a Plasmodium falciparum λgt11 library. The deduced protein sequence from cDNA clones revealed a high degree of homology to the rab/ypt family, which is involved in intracellular protein transport from the endoplasmic reticulum to the plasma membrane. A transcript of ~1.3 kb was detected in the sexual stage of gametes/zygotes, and not in the asexual erythrocytic stage of merozoites. The gametes/zygotes represent an extracellular population only found in the mosquito midgut. This finding suggests there may be other molecular components involved in the protein transport of intraerythrocytic stages of parasites.

I 366 THE SMALL GTP-BINDING PROTEIN RAC REGULATES LAMELLIPODIAL AND FILIPODIAL DYNAMICS AND CONTROLS CELL-SUBSTRATE ADHESION IN XTC FIBROBLASTS, Marc Symons, Rong-Guo Qiu, Robin Clark and Frank McCormick, Onyx Pharmaceuticals, Richmond, CA 94806

We have previously obtained evidence for the existence of a ras-like GTP-binding protein involved in the control of actin cytoskeleton and cell-substrate adhesion using microinjection of guanine nucleotide analogs in Xenopus XTC fibroblasts (J. Cell Biol. 118, 1235). We therefore investigated the role of rac, a GTP-binding protein involved in the regulation of plasma membrane ruffling. Injection of the constitutively active mutant rac2L61 in XTC fibroblasts inhibited filipodia and ruffling, induced the formation of lamellipodia and increased cell spreading. No alterations in stress fibers could be detected. Injection of a dominant negative rac mutant had opposite effects: it inhibited lamellipodia and cell spreading and stimulated formation of filipodia. These changes in cell morphology are independent of rho function, a GTP-binding protein which controls the formation of stress fibers, since pre-injection of C3 transferase did not inhibit the changes in cell morphology induced by rac2L61, although it strongly diminished the presence of stress fibers. Injection of rac2L61 also strongly inhibited cell rounding caused by GRGDSP peptides but had no effect on cell rounding caused by trypsin, indicating that activation of rac leads to an increase in cell-substrate adhesion. The effects of the various rac mutants suggest that rac may regulate lamellipodial and filipodial dynamics via its control of cell-substrate adhesion.

I 367 RAS ACTIVATION RESULTS IN CD69 EXPRESSION

Roberto Testi*^, Daniele D'Ambrosio*, Doreen Cantrell# and Angela Santoni*. *Dept. of Experimental Medicine, Univ. of Rome "La Sapienza", Italy, ^Dept. pf Experimental Medicine and Biochemical Sciences, Univ. of Rome "Tor Vergata", Italy, and #Lymphocyte Activation Laboratory, ICRF, London, UK.

Ras activation plays a central role for the expression of early genes involved in the progression of the activation process in lymphoid cells. We investigated the possible involvement of ras in the induction of the early activation receptor CD69 in T cells.

Jurkat T cells were transiently transfected with a mutated v-Ha-*ras*, coding for a constitutively active ras which could be immunoprecipitated mostly in GTP-bound form and could transactivate a cotransfected AP-1/CAT gene. In v-Ha-*ras*, but not in control vector transfected cells, CD69 was induced on the membrane after 18 hours at levels comparable to those induced by PMA treatment. Expression of other surface molecules was not affected. We asked therefore whether ras activation was necessary for TCR/CD3-mediated CD69 induction. Jurkat cells were transiently transfected with c-Ha-*ras*-N17, which codes for a negative dominant mutant of ras. In c-Ha-*ras*-N17 transfeted cells, PMA-induced association of ras with GTP was greatly reduced, as was also inhibited PMA-induced AP-1/CAT transactivation. To evaluate CD69 induction by FACS analysis in c-Ha-*ras*-N17 transfeted cells, Jurkat cells were cotransfected with a rat CD2 molecule devoid of cytoplasmic tail. After transfection, cells were stimulated through the TCR/CD3 by crosslinking antibodies, or with PMA, and CD69 expression was revealed by FACS analysis on both transfected (rat $CD2^+$) and non transfected (rat $CD2^-$) cells. In c-Ha-*ras*-N17 transfected cells both TCR/CD3 and PMA stimulation could not result in significant CD69 induction compared to untransfected cells in the same culture or cells effectively transfected with control vector.

These data indicate that ras activation is sufficient and mostly necessary for CD69 induction in Jurkat T cells, and suggest that CD69 expression may represent a useful tool to detect ras activation in T lymphocytes.

I 368 THE *Drosophila melanogaster* HOMOLOG OF THE NEUROFIBROMATOSIS TYPE 1 TUMOR SUPPRESSOR GENE, Inge The, Gregory E. Hannigan, James F. Gusella, Iswar K. Hariharan and Andre Bernards, Massachusetts General Hospital Cancer Center and Harvard Medical School, Building 149, 7th floor, 13th street, Charlestown, MA 02129.

Neurofibromatosis type 1 (NF1) is one of the most common genetic diseases in humans which primarily affects cells of neural crest origin. The gene that is mutated in NF1 patients has been cloned. Part of the protein has sequence homology to the mammalian *ras* GTPase activating protein (*ras*GAP) and to the yeast *ras*GAPs IRA1 and IRA2. It has been shown that this domain of NF1 increases the GTPase activity of *ras*.

In order to investigate the biological function of NF1 we have cloned the Drosophila homolog of NF1 (DNF1). The Drosophila and human NF1 proteins show a high level of sequence identity through the whole length of the protein (75% identity in the GAP related domain). The DNF1 gene has been mapped to the 96F region on the right arm of the third chromosome of the Drosophila genome.

Flies with heterozygous deletions that span the DNF1 transcription unit were indistinguishable from wild type flies and homozygous deletions are lethal. However, these deletions remove numerous genes. We have screened the 96F region for lethal mutations and have isolated 5 genetic complementation groups. At this moment, we are determining which of the complementation groups is a DNF1 mutant. Our goal is to use the DNF1 mutants to understand the role of NF1 in the *ras* signal transduction pathway, since this pathway seems to be conserved between human and Drosophila.

I 369 **FLUID SHEAR STRESS ACTIVATES MITOGEN-ACTIVATED PROTEIN KINASES IN ENDOTHELIAL CELLS: ROLE OF G-PROTEINS**

Hennessey Tseng and Bradford C. Berk, Cardiology Division, Emory University, Atlanta, GA 30322

Fluid shear stress is a powerful mediator of vessel function altering protein and gene expression. The signal transduction mechanisms stimulated by shear stress in endothelial cells are receptor-like and include activation of phospholipase C and mobilization of intracellular Ca^{2+}. However, the nature of the plasma membrane shear stress sensing mechanism remains unclear. To assay for endothelial cell responses to flow, we used stimulation of mitogen-activated protein kinase (MAPK) activity as an index. Flow activated MAPK in a force-dependent manner with peak activation at 2.0 dynes/cm^2 for 5 min. We found that flow-stimulation of MAPK was independent of Ca^{2+}. To determine whether a G-protein coupled event was involved, we inhibited GTP-binding protein function with the nonhydrolyzable GDP analog, GDP-βS. Cells were scrape loaded with GDP-βS (30-600 μM) and allowed to recover for 48-56 h. Flows were performed at 2.0 dynes/cm^2 for 5 min on early passage cells (passage < 6). Using a parallel plate flow chamber, we measured the activity of MAPK in bovine aortic endothelial cells. There was a concentration-dependent inhibition of MAPK activation as determined by phosphorylation-induced band shift on Western blot. To show that GDP-βS was effective, we demonstrated inhibition of α-thrombin activation of MAPK in a similar manner. These data establish the involvement of a GTP-binding protein in fluid shear stress signal transduction in endothelial cells. Furthermore, these findings imply that the shear stress sensor is a receptor-like protein.

I 370 RAB3 PROTEINS IN RBL2H3 CELL EXOCYTOSIS

Michael Tuvin, Robert Lampert, Wei Qien, Brian Knoll and Burton Dickey, Department of Pulmonary Medicine and Cell Biology, Baylor College of Medicine, Houston, TX 77030.

The rat basophilic leukemia (RBL2H3) cell line serves as a model for tissue mast cells that are thought to play a key role in initiating inflammatory diseases, including asthma. RBL2H3 cells undergo dramatic ligand-stimulated exocytosis, which may be assayed in single cells via patch clamping. Substantial evidence supports Rab3a involvement in synaptic vesicle release, and indirect evidence implicates Rab3d in insulin-induced exocytosis in adipocytes. We therefore hypothesized that a Rab3 isoform regulates stimulated exocytosis in RBL2H3 cells.

We designed Rab3-specific PCR primers which averaged 86% identity with the four known Rab3 cDNAs (a-d), but only about 40% with irrelevant Rabs. PCR amplification of RBL2H3 reverse transcripts produced an expected 370 bp fragment of DNA. Subsequent cloning and bidirectional sequencing unexpectedly yielded several Rab3-isoforms; namely Rab3a, Rab16(3d)*, and an as yet unpublished rat homologue of human Rab3b (98% identity at the amino acid level). We are now using the cDNA fragments as probes for cDNA screening of the RBL2H3 library, in an attempt to recover full length Rab3 clones.

We also have designed a set of antisense oligonucleotides specific to each of the four Rab3 isotypes. Since regulated exocytosis is not essential to cell survival, loss of Rab3 function should not result in cell death or major phenotypic changes other than the loss of stimulated exocytosis. Both single cell and population based assays of ligand-stimulated exocytosis in antisense-treated RBL2H3 cells are in progress. Through these experiments, we plan to explore the mechanism of stimulated exocytosis by myeloid cells.

*Despite its numeric assignment, Rab16 appears to be a Rab3 isoform. Our sequencing revealed a 1 bp deletion in the reported Rab16 sequence, which created a mismatch of 12 amino acid residues when compared with other known Rab3s. An additional downstream insertion of 1 bp restores the frame, making it difficult to recognize the error. Amino acid identity of the corrected sequence with mouse Rab3d is 98%.

I 371 RAS SIGNALING AND GLUCOSE TRANSPORT IN 3T3-L1 ADIPOCYTES.

N. van den Berghe[1], D.M.Ouwens[2], J.A.Maassen[2], M.G.H. van Mackelenbergh[1], H.C.M.Sips[1] and H.M.J.Krans[1].
[1] Dept. of Endocrinology; University Hospital Leiden.
[2] Dept. of Medical Biochemistry, The Sylvius Institute, Leiden, The Netherlands.

At present it is unknown which signals are involved in insulin-mediated glucose transport. The role of ras in this process is controversial. We therefore investigated the role of ras signaling in glucose transport by examining the effects of thrombin, EGF and insulin on ras-GTP formation and MAP kinase activation in relation to their ability to stimulate glucose transport in 3T3-L1 adipocytes. Thrombin, EGF and insulin were chosen as they are known to activate ras/MAP kinase in a variety of cell types. Results: In 3T3-L1 adipocytes the three agonists stimulated the formation of ras-GTP transiently with a peak at 5-10 minutes. However, only thrombin was found to induce a second peak after 30 minutes. The activation of ras was paralleled with both the phosphorylation and activation of MAP kinase: transient for insulin and EGF, biphasic for thrombin, in concordance with ras being an upstream regulator of MAP kinase. In contrast, glucose transport was only stimulated by insulin and not by EGF or thrombin. These results indicate that activation of ras and MAP kinase do not trigger glucose transport. However, it is still possible that other signals are needed in conjunction with ras/MAP kinase to induce glucose uptake, which are activated by insulin and not by EGF or thrombin. In addition, we cannot exclude the possibility that MAP kinase is regulated differently when activated by insulin, thrombin or EGF. In that regard, we have preliminary data that protein kinase C has opposing effects on MAP kinase activation by insulin compared to EGF and thrombin. Hypothetically, this could result in the specific phosphorylation of proteins involved in glucose transport by MAP kinase in response to insulin, but not by EGF or thrombin.

I 372 MAP KINASE ACTIVATION DOWNSTREAM FROM p21 ras IS NOT REQUIRED TO PROMOTE SURVIVAL OF RAT SYMPATHETIC NEURONS, K. Virdee & AM Tolkovsky, Department of Human Anatomy, University of Oxford, South Parks Road, Oxford OX1 3QX (UK).

Sympathetic neurons from neonatal rats depend on nerve growth factor (NGF) for their survival both *in vivo* and in culture. When NGF is withdrawn these neurons die by activating a cell death programme. We have found that the survival by each of the following; NGF, which activates the tyrosine kinase receptor trk, the cytokines LIF and CNTF, which bind to a common set of receptors that do not contain intrinsic tyrosine kinase activity, and the cyclic AMP analogue CPTcAMP, which activates intracellular PKA, is blocked by injection of the FAB fragments of the anti-ras antibody Y13-259. These results have suggested that activation of ras is critical for survival. As there is strong evidence indicating that the activation of MAP kinases is an event downstream from ras activation, we haye examined whether each of these survival factors cause MAP kinase activation. While all agents (NGF, LIF, CNTF and CPTcAMP) were able to promote survival of rat sympathetic neurons in culture, we have found that only NGF was able to cause a robust (> 100 fold) and persistent (to 24h) activation of two MAP kinase species; ERK1 and a 45-46 kDa ERK). By contrast, LIF, CNTF and CPTcAMP were unable to induce detectable activation of MAP kinase although each agent was able to affect survival for several days (the cytokines) or weeks (cAMP analogue). One clear difference between these factors is that NGF causes significant neuronal hypertrophy compared to the other agents, suggesting that MAP kinase activation may be an adjunct in enhancing protein synthesis. Studies will be described which aim to establish whether the activation of MAP kinase is an obligatory requirement for the NGF-induced survival pathway. Our results suggest that there are at least two divergent pathways downstream from ras in sympathetic neurons: 1) a ras-MAP kinase inducible pathway (which may be linked to activation of protein synthesis and gene expression) and 2) a ras-X (kinase) pathway. It is our contention that the latter pathway is crucial for neuronal survival.

I 373 Tyrosine kinase signalling during early Xenopus development. Malcolm Whitman, Carole LaBonne, Hui-Chuan Huang, Don Slish. Department of Cell Biology, Harvard Medical School Boston MA 02115

We are currently investigating the signal transduction mechanisms by which FGF and activin induce mesoderm in early Xenopus prospective ectoderm. We have used both anti-phosphotyrosine antibodies and SH2 domain containing proteins as affinity reagents to investigate regulation of cellular tyrosine kinase substrates in response to mesoderm inducers. We have identified MAP/ERK kinase as a rapid, major target for FGF stimulation of early blastomeres, and are currently characterizing additional potential tyrosine kinase substrates. Using overexpression of dominant inhibitory mutant of components of the FGF signaling pathway, we have found that activin signaling is dependent on FGF signaling. We also find that activation of any of several downstream targets for FGF receptor signaling (e.g. ras, raf) is sufficient to rescue cellular responsiveness to activin in the presence of a dominant inhibitory FGF receptor. We are currently examining the basis for this requirement. In addition, we are examining point mutants in the phosphotyrosine SH2 recognition domains of protein tyrosine kinases to determine which, if any, of these sites are important for the transmission of inductive signals.

I 374 COMPARISON OF HUMAN CAP AND CAP2, HOMOLOGS OF THE YEAST ADENYLYL CYCLASE-ASSOCIATED PROTEINS. **Dallan Young, John Swiston and Gang Yu, Department of Medical Biochemistry, University of Calgary Health Science Centre, Calgary, Alberta, T2N 4N1, Canada.**

We previously reported the identification of a human cDNA encoding CAP, a protein that is related to the *S. cerevisiae* and *Sz. pombe* adenylyl cyclase-associated CAP proteins. We have cloned cDNAs encoding a second human CAP related protein, CAP2. Human CAP and CAP2 are 64% identical. Previous work has shown that *S. cerevisiae* CAP has two distinct functional domains: the N-terminal domain is required for hyper-activation of adenylyl cyclase by $RAS2^{val19}$, while loss of the C-terminal domain results in morphological and nutritional defects that appear to be unrelated to the cAMP pathway including abnormal morphology and actin distribution, random budding, and growth sensitivity to temperature and nutritional conditions. Our complementation studies show that human CAP and CAP2 share conserved functional properties with the C-terminal domains of *S. cerevisiae* and *Sz. pombe* CAP proteins. In contrast, expression of either human CAP or CAP2 in *S. cerevisiae cap*$^-$ strains does not restore responsiveness of adenylyl cyclase to $RAS2^{val19}$. However human CAP, but not CAP2, does suppress the propensity to sporulate due to deletion of the N-terminal domain of CAP in *Sz. pombe*, suggesting that it restores normal adenylyl cyclase activity.

I 375 THE DROSOPHILA *ROLLED* LOCUS ENCODES A MAP KINASE REQUIRED IN THE SEVENLESS SIGNAL TRANSDUCTION PATHWAY, Kenton Zavitz, Xinghong Dong, William Biggs, and Larry Zipursky, Department of Biological Chemistry, UCLA School of Medicine and Howard Hughes Medical Institute, Los Angeles, CA 90024-1662.

The development of the R7 photoreceptor cell in the *Drosophila* retina requires an inductive interaction between the sevenless (sev) receptor tyrosine kinase, located on the surface of the R7 precursor cell, and the bride-of-sevenless (boss) protein expressed on the surface of the neighboring R8 photoreceptor cell. Ras1, putative regulators of Ras1 (Gap1, Sos, and Drk), and the serine/threonine kinase Raf-1 have been shown to be components of the sev signal transduction pathway. Alleles of *rolled(rl)* act as suppressors of an activated form of Draf1. We have shown both molecularly and genetically that the *rl* locus encodes a Drosophila MAP kinase (ERK-A) which functions downstream of Raf-1 in R7 induction.

Transgenic flies were generated that express either active ($ERK\text{-}A^{WT}$) or inactive ($ERK\text{-}A^{K164}$) forms of the ERK-A protein under the control of both the *sev* enhancer, driving expression within the R7 equivalence group, and the heat shock inducible *hsp70* promoter. The $ERK\text{-}A^{WT}$, but not $ERK\text{-}A^{K164}$, substituted for *rl* in three different genetic tests: 1) The suppression of the activated $Draf^{tor4021}$ rough eye phenotype caused by removing one copy of rl was reversed by $ERK\text{-}A^{WT}$; 2) $ERK\text{-}A^{WT}$ also reversed the suppression of the sev^{E4}; Sos^{JC2} phenotype caused by removal of one copy of *rl* ; and 3) Periodic heat shock-induced expression of $ERK\text{-}A^{WT}$ throughout development rescued the larval lethality of *rl* null alleles. These studies provide the first genetic evidence of a requirement for MAP kinase in signaling downstream of receptor tyrosine kinases.

In an attempt to identify targets downstream of MAP kinase during R cell development, we have taken two approaches. Flies homozygous for the weak *rl* allele, rl^1, have rough eyes due to approximately 2/3 of their ommatidia containing fewer R cells, with the development of R7 appearing to be most sensitive to a reduction in *rl* activity. In a screen of deficiencies covering the third chromosome, several loci acting as dominant enhancers or suppressors of this rough eye phenotype have been identified. In addition, we have utilized a yeast interaction-trap selection from an imaginal disc cDNA library to identify proteins which interact directly with MAP kinase during eye development. Results from these two screens will be presented.

I 376 THE CDC25Mm PRODUCTS CORRESPONDING TO FORM I AND IV EXHIBIT A DIFFERENT SERUM-DEPENDENT RAS ACTIVATION., R.Zippel, E. Martegani, M.Vanoni, E.Sturani. Dipartimento di Fisiologia e Biochimica Generali Università degli Studi di MIlano, 20133 MIlano, Italy.

Four different cDNA full lenght codified by a single gene have been identified for the $CDC25^{Mm}$ Guanine Nucleotide Exchange Factor in mouse. All of them encode for a common open reading frame which comprises a catalytic C-terminal domain with exchange activity toward ras but differ in lenght of the N-terminal domain. Moreover they diverge in the 5' non coding region suggesting that these products could have been raised from an alternative splicing process. (Cen et al. EMBO J. **11**, 4007, 1992)

The longest form ($CDC25^{Mm}IV$) encodes for a protein of 140 kDa. which shows in the N-terminal domain a pleckstrin homology region (PH) and a dbl homology region, while the shortest cDNA ($CDC25^{Mm}I$), which encodes for a protein of 661 aa. is devoid of those homology regions.

Experiments were undertaken to gain information on the role of the N-terminal domain of $CDC25^{Mm}IV$ in modulating ras activity. Our results indicate that both $CDC25^{Mm}I$ and ^{Mm}IV are able to transactivate a ras responsive element but while $CDC25^{Mm}I$ acts in a costitutive way, a modulation of $CDC25^{Mm}IV$ activity on ras was observed after serum stimulation.The effects of different growth factors on $CDC25^{Mm}IV$ activity will be presented.

Poster Session IV

I 400 TYROSINE KINASES (PYKs), TYROSINE PHOSPHATASES (PYPs) AND THE ACTIONS OF G-PROTEIN-COUPLED CONTRACTILE AGONISTS IN VASCULAR AND GASTRIC SMOOTH MUSCLE, Sultan Ahmad, Mahmoud Saifeddine, Adebayo Laniyonu, Yasuo Oda and Morley D. Hollenberg, Endocrine Research Group, Departments of Pharmacology & Therapeutics and Medicine, University of Calgary, Faculty of Medicine, Calgary, AB Canada T2N 4N1

Based on our initial observations that genistein (GS) and tyrphostin (TP) can selectively block the contractile actions of angiotensin-II (A-II) in gastric smooth muscle (GM) (J. Pharmacol. Exp. Ther. 264, 958), we have begun to study the roles of PYKs, PYPs and their substrates in the control of G-protein-coupled agonist action in both gastric (GM) and vascular (VM) contractile preparations. As in the GM preparations, in VM, both GS and TP can selectively block the contractile actions of A-II and other G-protein-coupled agonists, whereas vanadate (VAN) not only potentiates agonist action but exhibits contractile activity on its own. Further, A-II and VAN cause a GS-blockable transient increase in VM and GM phosphotyrosyl proteins detected by Western blot. In the VM tissue we also detect TP and GS-inhibited src-related PYK activity that can be resolved by column chromatography; and we have PCR-cloned a number of distinct PYKs and PYPs from VM-derived cell cultures. We conclude that the PYKs and PYPs present in vascular and gastric smooth muscle may play a dynamic role in the control of both resting tension and G-protein-coupled agonist-triggered contraction. (Supported by the Canadian MRC, the Alberta Heart and Stroke Foundation and the Canadian Diabetes Association).

I 401 ERYTHROPOIETIN ACTIVATES THE TYROSINE PHOSPHORYLATION OF SHC WHICH COUPLES TO THE RAS SIGNALING PATHWAY.
Dwayne L. Barber and Alan D. D'Andrea. Divisions of Cellular and Molecular Biology and Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, 02115, U.S.A.

The erythropoietin receptor (EPO-R), a member of the cytokine receptor family, signals both mitogenic and differentiative pathways when expressed in erythroid progenitor cells. We have utilized a murine interleukin-3 (IL-3) dependent cell line, Ba/F3, and a murine IL-2 dependent cell line, CTLL, transfected with the EPO-R cDNA to examine signal transduction. Activation of the EPO-R by either EPO or the Friend Spleen Focus-Forming Virus (SFFV) glycoprotein, gp55, results in the tyrosine phosphorylation of EPO-R and JAK-2. A dominant negative EPO-R, [EPO-R(T)] which lacks the cytoplasmic region of the EPO-R, blocks these phosphorylation events and inhibits mitogenesis. In this study we have shown that the Src homology 2 (SH2) adapter protein, Shc, is tyrosine phosphorylated in response to IL-3 or EPO in Ba/F3-EPO-R cells, and by IL-2 or EPO in CTLL-EPO-R cells. The phosphorylation of Shc is observed by 1 min, peaks from 5-15 min and subsides by 30 min. In addition, Grb2 and a tyrosine phosphorylated protein of 145 kDa associates with Shc in a time-dependent fashion. Factor-independent Ba/F3-EPO-R-gp55 cells also show constitutive phosphorylation of Shc. In cells which co-express the full length EPO-R and the dominant negative EPO-R(T), EPO fails to induce the tyrosine phosphorylation of EPO-R, JAK-2 and Shc. In conclusion, in signal transduction by the EPO-R, Shc may act as the primary adapter protein for linking to the Ras pathway in hematopoietic cells.

I 402 THE RECEPTOR TYROSINE-KINASE *ark*, A MEMBER OF THE IMMUNOGLOBULIN SUPERFAMILY, CAN MEDIATE CELL-CELL AGGREGATION BY A HOMOPHILIC BINDING MECHANISM. Paola Bellosta, Debby A. Lin and Claudio Basilico, Department of Microbiology, New York University School of Medicine, New York, NY 10016.

Receptor tyrosine kinases (RTKs) are important in mediating signals controlling cell growth and differentiation. From a mouse cDNA library we isolated a tyrosine kinase receptor (ARK= adhesion-related kinase) whose deduced amino acid sequence reveals in the extracellular domain a combination of motifs from the immunoglobulin and fibronectin typeIII superfamilies. *In situ* hybridization analysis, on adult mouse brain, reveals a high level of expression of *ark* mRNA in the dentate gyrus and hippocampus as well in the cerebellar cortex. The molecular basis of cell-cell contact mediated by members of the Ig and FNIII superfamilies is known to involve protein-protein interaction, with either soluble or cell bound molecules, through homophilic and/or heterophilic binding mechanisms. In order to study the ability of ARK to mediate homophilic interaction, we performed aggregation experiments using either CHO cells or *Drosophila* S2 cells, transfected with the full-length *ark* cDNA. These experiments show that ARK receptor promotes cell-adhesion in a homophilic, Ca^{++} independent manner leading to activation of its tyrosine kinase. Using a construct in which the entire tyrosine-kinase domain was deleted we were able to show that tyrosine phosphorylation is not required for the aggregation. Signaling events regulated by the extracellular matrix (ECM) and adhesion receptors may involve tyrosine phosphorylation as has been shown in the case of integrins or cell adhesion molecules. Our results suggest that ARK receptor may function as a cellular adhesion molecule by binding to a transmembrane molecule, and could represent a link between RTKs and cell adhesion molecules like integrins or neuronal adhesion molecules.

I 403 SUBCELLULAR DISTRIBUTION, PROTEIN ASSOCIATION, AND CSF-1-INDUCED TYROSINE PHOSPHORYLATION AND ACTIVATION OF HEMATOPOEITIC CELL PHOSPHATASE PTP-1C, Karen L. Berg, Douglas Einstein, Y. G. Yeung, and E. Richard Stanley, Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY 10461.

Recent studies in our lab (Yeung, et al, 1992) have demonstrated that the hematopoietic cell protein tyrosine phosphatase, PTP-1C is phosphorylated on tyrosine in response to the macrophage growth factor, Colony Stimulating Factor-1 (CSF-1). Further studies were undertaken to determine the effect of growth factor stimulation and the resulting tyrosine phosphorylation on the subcellular distribution of PTP-1C, the association of PTP-1C with other tyrosine phosphorylated proteins and the phosphatase activity of PTP-1C. PTP-1C, which is predicted to be a cytosolic protein, was found to reside in both the cytoplasmic and membrane fractions at a ratio of approximately 80:20. Tyrosine phosphorylated PTP-1C was found to accumulate in both the cytosol and membrane, however there is no detectable movement of PTP-1C between the two locations. An immune complex phosphatase assay demonstrates that tyrosine phosphorylation of PTP-1C is associated with an increase in its *in vitro* specific activity, suggesting that growth factor induced tyrosine phosphorylation activatesPTP-1C. Co-immuneprecipitation experiments suggest that membrane bound PTP-1C associates with a 145 kDa tyrosine phosphorylated protein, both in the absence and presence of growth factor, and may explain the presence of PTP-1C in membrane fractions, since it has no predicted membrane spanning sequences. Growth factor treatment appears to cause a marginal increase in the tyrosine phosphorylation of the PTP-1C associated145 kDa protein, and this protein is dephosphorylated in an in vitro PTP-1C immune complex phosphatase assay, suggesting the possibility that it could be one of the biological substrates of PTP-1C. Interestingly, PTP-1C does not appear to associate with the activated CSF-1R, in contrast to the related, ubiquitously expressed phosphatase SH-PTP2 which has been demonstrated to associate with the activated PDGF and EGF receptors.

I 404 THE CLONING OF A cDNA ENCODING A PUTATIVE EMBRYONIC AND CNS TRANSMEMBRANE RECEPTOR TYROSINE KINASE. Leslie G. Biesecker[1], Diane Giannola[2], and Stephen G. Emerson[2]. [1]NIH-National Center for Human Genome Research, and [2]Department of Internal Medicine, University of Michigan School, Ann Arbor, MI, 48109.
A growing list of growth factors and cytokines have been implicated in the differentiation of the mammalian embryo. Several of these ligands mediate their functions through receptors that belong to the protein kinase family. To characterize the molecular regulation of early mammalian development we have isolated additional members of this class of kinases from embryonic tissue. We have made use of the differentiation potential of the murine embryonic stem cell system to clone a cDNA that encodes a putative embryonic receptor tyrosine kinases (ETK-2). This cDNA is one of four protein kinases cloned from in vitro differentiated embryonic stem cells using PCR with degenerate oligonucleotide primers (Biesecker, et al PNAS v90 7044-7048, 1993). The partial (141 base) clone in the above report has been extended to 3.71 kb by library screening with the original PCR product as a probe. The cDNA encodes an open reading frame of 947 amino acids and contains a protein kinase catalytic domain, a transmembrane domain and an extracellular domain. The ETK-2 transcript is expressed in adult brain and testicle and is approximately 4.4 kb. We have also demonstrated expression in undifferentiated and differentiated ES cells and in a number of hematopoietic and non-hematopoietic tumor cell lines. High titer polyclonal antibodies have been raised to a peptide synthesized from the derived cDNA sequence and immunofluorescence studies will be presented. Further studies will include confirmation of the 5' mRNA terminus, genomic cloning, and in vitro demonstration of tyrosine kinase activity.

I 405 CHARACTERIZATION OF THE PROMOTER REGION OF STROMELYSIN, A MATRIX METALLOPROTEINASE INVOLVED IN ARTHRITIS, Paula Borden, Nancy Fan, and Renu Heller. Syntex Research, 3401 Hillview Ave., Palo Alto, CA 94304
Matrix metalloproteinases (MMP's) are a family of enzymes with important roles in bone remodeling during development, but are also found in certain pathological conditions such as joint destruction that occurs in arthritis. In human fibroblasts, stromelysin expression has been shown to be induced by inflammatory agents IL-1 and TNF, tumor promoter TPA, growth factors such as PDGF, and oncogenes, and suppressed by dexamethasone or TGF-ß. We have made reporter gene constructs with the -280 to +50 region of the stromelysin gene driving lac Z expression, and made stable cell lines with these constructs. In an osteosarcoma cell line U2OS, this construct is inducible weakly with IL1α and TNF but strongly with TPA; in a second osteosarcoma line MG63, this promoter is strongly induced by IL1α and TNF but not by TPA, suggesting that the signal transduction pathways governing expression of stromelysin in response to IL1α and TNF vs. TPA are distinct. Gel-shift assays and DNAse footprinting are being conducted to further elucidate the control mechanisms involved. During the course of our analyses of the stromelysin promoter, we detected a previously undescribed sequence of approximately 500 bp at a sited corresponding to -480 in the published sequence. This segment appears to form secondary structure. We have shown that this segment is indeed present in the human genome and are determining what effect it has on stromelysin gene expresssion.

I 406 ANALYSIS OF THE SIGNAL TRANSDUCTION PATHWAY TRIGGERED BY TRK PROTO-ONCOGENE (NGF RECEPTOR) AND BY ITS ONCOGENIC VERSIONS, M.G. Borrello, P.G. Pelicci*, G. Pelicci*, M.A. Greco, E. Arighi, L. Alberti and M.A. Pierotti - Istituto Nazionale Tumori, Milano and *Istituto Clinica Medica I, Policlinico Monteluce, Universita' di Perugia, Perugia.

We have detected TRK oncogene activation in 8 out of 52 cases of human papillary thyroid carcinomas. TRK oncogenes are created by chromosomal rearrangements fusing the TK domain of proto-TRK gene, encoding one of the receptor for nerve growth factor, to unrelated sequences.The sequences rearranged with proto-TRK were tropomyosine in three cases, tpr in three cases and a novel gene in one case. The three different oncogenic trk chimeric proteins were identified by Western blot using a specific antiserum; in addition they were demonstrated to be constitutively phosphorylated on tyrosine as the NGF activated proto-trk receptor protein. We began to study the signal transduction triggered by the trk oncoproteins in NIH/3T3 cells and compared it to that triggered by the proto-oncogene product activated by NGF.
Trk oncoproteins, as well as activated proto-trk product, bind in vitro the SH2 domain of the adaptor proteins Grb2 and Shc and both the SH2 domains of PLC-gamma. Moreover, in living cells, shc proteins are phosphorylated on tyrosine and coimmunoprecipitate with Grb2, with PLC-gamma and with trk and proto-trk proteins. Studies with other signal transducer proteins are in progress.

I 407 CLA-1: A NOVEL CYCLOPHILIN LIGAND CAN ACTIVATE THE CALCIUM SIGNAL-TRANSDUCTION PATHWAY LEADING TO IL2 TRANSCRIPTION, Richard J. Bram, Department of Experimental Oncology, St. Jude Children's Research Hospital, Memphis, TN 38101.
The immunosuppressant drug cyclosporin A (CsA) is thought to block T cell activation by inhibiting the calcium-activated protein-phosphatase calcineurin. CsA binds with nanomolar affinity to the intracellular protein cyclophilin, and confers upon it the ability to form a stable inhibitory complex with calcineurin. It is possible that endogenous regulatory pathways also use this mechanism to activate or repress T cell function.
A human B-cell cDNA library was screened using the yeast two-hybrid system to identify proteins that can bind to human cyclophilins A or B. Potentially interacting clones were subsequently tested for ability to regulate activation of NFAT transcriptional activity. One clone (CLA-1, for Cyclophilin Ligand that Activates) was found to activate NFAT-specific transcription following overexpression in Jurkat cells. The activation required phorbol ester (PMA), but did not require calcium ionophore, suggesting that it can replace the calcium flux requirement for NFAT-specific transcription. Overexpression of CLA-1 in the presence of PMA also activated transcription from an OAP/Oct enhancer-promoter construct and from the intact IL-2 enhancer, but did not affect AP-1 activity. CLA-1 induced NFAT-activation could be inhibited by low levels of CsA (50 ng/ml) or FK506 (0.5 ng/ml), suggesting that it acts directly or indirectly on calcineurin.
The CLA-1 message is approximately 1 kb in length, and has a single open reading frame which is predicted to encode a polypeptide of 297 amino acids, with a calculated MW of 33 kDa. CLA-1 may be a normal component of the signal transduction cascade in lymphocytes or other cells, and may present a novel target for immunosuppressive strategies. Furthermore, the finding of a putative cyclophilin-ligand that can mediate T-cell activation implicates cyclophilins in the process of signal transduction, even in the absence of CsA.

I 408 MOLECULAR CHARACTERIZATION OF A NOVEL, STATHMIN-LIKE NEURON SPECIFIC PHOSPHOPROTEIN P60,

Byk, T., Dobransky, T. Maucuer, A. and Sobel, A., INSERM U153, 17, rue du Fer à Moulin, 75005 Paris, FRANCE

P60 is a neuron specific phosphoprotein first identified by its interaction with an antiserum prepared against stathmin, a ubiquitous, evolutionary well conserved phosphoprotein which seems to act as an intracellular relay integrating various extracellular signals involved in cell regulation. Like stathmin, P60 exists in at least one unphosphorylated and several phosphorylated forms which can all be reduced to one unphosphorylated form upon dephosphorylation by alkaline phosphatase. Antibodies prepared specifically against P60 recognize additional forms of this protein with a molecular weight around 60-70kDa.

P60 is an *in vitro* substrate for casein kinase II, cdc2 and cAMP kinase. It is mainly expressed in neonatal brain where its concentration declines during adult development.

In vivo phosphorylation experiments in PC 12 cells show that at least one phosphorylated form of P60 disappears in favour of a less phosphorylated form in response to induction of neuronal differentiation with NGF, indicating that, like in stathmin, the phosphorylation level of this protein depends on the state of activation and differentiation of the cell.

After partial purification of P60 from neonatal mouse brain, the aminoacid sequences of several internal peptides could be determined. Oligonucleotides based on the primary sequences were used to screen a neonatal mouse brain Uni-Zap cDNA library. Sequence determination from the positive clones identified will give further insight on the identity and the molecular features of P60, as well as on its molecular relationship to stathmin.

I 409 INTEGRAL MEMBRANE GLYCOPROTEIN ACTIVATOR FOR $p185^{neu}$: A MECHANISM FOR AUTO-ACTIVATION OF ASCITES TUMOR CELLS. Kermit L. Carraway,[1] Dalia Lorenzo,[2] Pamela M. Guy,[3] Maria E. Carvajal,[1] Richard A. Cerione,[3] Kermit L. Carraway III,[3] and Coralie A. Carothers Carraway[2], Departments of [1]Cell Biology & Anatomy and [2]Biochemistry & Molecular Biology, University of Miami School of Medicine, Miami, FL 33101, and [3]Department of Pharmacology, Cornell University, Ithaca, NY 14853

The proto-oncogene $p185^{HER2/neu}$ is a cell surface receptor tyrosine kinase related to the EGF receptor. Although it has been implicated in breast cancer and other carcinomas, the nature and role of its activating ligand(s) have not been defined. We have cloned and sequenced ASGP-2, an integral membrane glycoprotein of the highly malignant 13762 ascites rat mammary adenocarcinoma. The deduced sequence contained two EGF-like domains having homology to EGF domains of active EGF-like proteins. Immunoblot analyses showed that microvilli from the ascites tumor cells do not contain the EGF receptor but do contain $p185^{neu}$. To determine whether ASGP-2 is an activator of p185, we used DHFR/G8 cells, 3T3 cells which have been transfected with *c-neu* to a high level but are not transformed. Addition of purified ASGP-2 to DHFR/G8 membranes caused an activation of p185 autophosphorylation and of membrane tyrosine kinase activity. Immunoblot analyses of the ASGP-2-treated membranes using anti-phosphotyrosine showed that p185 was the predominant tyrosine-phosphorylated protein in the membranes. ASGP-2 and p185 were co-immunoprecipitated from the treated membranes solubilized in Triton X-100 or RIPA buffer, indicating that they are present in a stable complex in the ASGP-2-treated DHFR/G8 membranes. To determine whether ASGP-2 and p185 are also associated in a complex in 13762 cells, microfilament-depleted microvillar membranes were isolated from the MAT-C1 ascites subline of the 13762 tumor, solubilized in nonionic detergent and fractionated by density gradient centrifugation to remove insoluble complexes. ASGP-2 and p185 can be co-immunoprecipitated from the soluble density gradient fractions. Autophosphorylation of p185 in the gradient fractions could be demonstrated with ^{32}P-ATP, indicating that p185 is active. Finally, ASGP-2 and p185 were co-immunoprecipitated from crosslinked microvilli after solubilization in sodium dodecyl sulfate and dilution into RIPA buffer. These results clearly show that ASGP-2 is an activator of $p185^{neu}$ and that p185 and ASGP-2 are present in a stable complex in the 13762 cells. The presence of this complex suggests that ASGP-2 is the native ligand for $p185^{neu}$ in these tumor cells. Since the ascites cells do not form cell-cell associations, we propose that formation of this intramembrane complex provides a mechanism for autonomous stimulation of these highly malignant and metastatic tumor cells.

I 410 Calcineurin: Molecular analysis of its interaction with drug-immunophilin complexes and its role in the regulation of NFAT. Neil A. Clipstone, David Fiorentino and Gerald R. Crabtree. Howard Hughes Medical Institute, Stanford Univ, Stanford CA 94305.

The immunosuppressive drugs CsA and FK506 block T cell activation by preventing the activation of transcription factors, such as NF-AT and NF-IL2A, that are involved in lymphokine gene expression. The inhibitory effects of CsA and FK506 are manifest via interaction with their cognate intracellular receptors cyclophilin and FKBP (collectively immunophilins). *In vitro* studies have demonstrated that drug-immunophilin complexes bind the calcium/calmodulin regulated phosphatase, calcineurin (PP2B) and inhibit its enzymatic activity. In this study, we have investigated the molecular requirements that govern the interaction of calcineurin with drug-immunophilin complexes. Calcineurin is comprised of a catalytic subunit (CNA) and a calcium binding regulatory subunit (CNB). We have mapped amino acid residues of the CNA subunit that are required for the interaction of calcineurin with drug-immunophilin complexes and have identified a 28 amino acid domain of CNA that forms the binding site for CNB. Furthermore, we demonstrate that the CNB subunit is absolutely required for the interaction between calcineurin and drug-immunophilin complexes. Recent studies *in vivo* have established calcineurin as a key signalling enzyme in the T cell signal transduction cascade and an important effector of the calcium signalling pathway leading to lymphokine gene expresion. Here we extend these studies by using a calcium-independent constitutively active calcineurin mutant to further probe the role of calcineurin in the regulation of the T cell specific transcription factor NFAT.

I 411 EXPRESSION OF RECEPTOR-TYPE PROTEIN TYROSINE PHOSPHATASES IN HUMAN BRAIN TUMORS. Charles A. Conrad, Huai Lin, W. K. Alfred Yung, and Peter A. Steck, Department of Neuro-Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030

Growing evidence suggests that protein tyrosine phosphatases (PTPases) are intimately involved in cellular growth signaling and differentiation through modulation of phosphorylation of important regulatory sites on proto-oncogenes. The expression of twelve soluble and receptor-type protein-tyrosine phosphatases (PTPases) were examined in various types of human brain tumors. The receptor-type PTPases revealed a differential expression, while the soluble forms showed consistent expression. In particular, HPTPase-α and HPTPase-β were highly expressed in rapidly proliferating medulloblastomas and glioblastomas multifomes. The other PTPases revealed variable expression among the tumors and tumor types. Since HPTP-α has recently been reported to actively dephosphorylate the negative regulatory src-527 site *in vitro*, possibly indicating it's role in cellular growth regulation We further assayed its expression in brain tumor cell lines. Functional and non-functional full length forms of HPTP-α were PCR cloned and inserted to a bacterial protein expression vector. Recombinant HPTP-α fusion proteins were isolated and purified to homogeneity. These functional and non-functional proteins were assayed for their ability to dephosphorylate src-527 compared to other phosphorylation sites. Polyclonal antibodies were also generated against the functional form of r-HPTP-α. The roles of the HPTPase-α in growth regulation was further examined in brain tumor cells lines utilizing specific antisense oligonucleotides against HPTPase-α. These results strongly implicate an inverse relationship between the expression of HPTPase-a and cellular proliferation in human brain tumors.

I 412 MOLECULAR CLONING OF *TRK*E, A NOVEL TYROSINE KINASE RECEPTOR ISOLATED FROM NORMAL HUMAN KERATINOCYTES, WHOSE PROLIFERATION IS POTENTLY STIMULATED BY NERVE GROWTH FACTOR. Michele De Luca, Eddi Di Marco, Nunzio Cutuli, Liliana Guerra, Sergio Bondanza and Ranieri Cancedda, Laboratorio di Differenziamento Cellulare, Istituto Nazionale per la Ricerca sul Cancro, 16132, Genova, Italy.

Normal human keratinocytes synthesize and secrete biologically active NGF in a growth regulated fashion. The same human keratinocytes bind NGF via low and high affinity receptors. In parallel with the course of NGF synthesis, the expression of low affinity NGF receptor ($p75^{NGFr}$), present only in the epidermal basal layer, decreases when a confluent, differentiated and fully stratified epithelium is obtained. The *trk*A protooncogene product ($p140^{trkA}$), a component of the NGF receptor, is not expressed by keratinocytes. Instead, keratinocytes express a new member of the *trk* gene family (that we termed *trk*E) which has been cloned from both keratinocytes and human fetal brain. Its open reading frame codes for a polypeptide of 876 amino acids exhibiting the classic features of cell surface tyrosine protein kinases. *Trk*E catalytic domain is 41% identical to *trk*A and shows several features unique to the *trk* gene family, while its extracellular domain does not show significant homology to any known proteins. *Trk*E generates 3.9 kb transcripts in normal human keratinocytes and in a variety of normal human tissues, and it is the first member of this gene family found abundantly and widely expressed in humans.

Several lines of indirect evidence suggest that NGF is the ligand for *trk*E: i) keratinocytes bind NGF with high affinity, ii) NGF potently stimulates keratinocyte growth in an autocrine fashion (direct effect of NGF on [^{3}H]thymidine incorporation, inhibition of autocrine keratinocyte growth by moAbs inhibiting human NGF biological activity and by a *trk*-specific inhibitor, the natural alkaloid K252a), iii) NGF exerts its biological effect on keratinocytes through the stimulation of a *trk* specific tyrosine kinase, iv) keratinocytes lack *trk*A but do express large amount of *trk*E.

I 413 ISOLATION OF TWO NOVEL STRUCTURALLY RELATED PROTEIN TYROSINE PHOSPHATASES FROM *XENOPUS LAEVIS* Robert Del Vecchio and Nicholas Tonks, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Reversible tyrosine phosphorylation is involved in diverse physiological events such as signal transduction processes controlling the cell cycle and early development. Protein tyrosine phosphatases (PTPases) are critical in regulating the cellular level of tyrosine phosphorylation. We have chosen *Xenopus laevis* as a model system to investigate how PTPases function in cellular signalling. Degenerate primers, corresponding to conserved residues within the catalytic domain of PTPases, were used in a PCR based protocol to isolate cDNA's representing 14 distinct phosphatase domains from *X. laevis* . Two of these cDNA's, designated X1 and X10, were chosen for further study and full length clones were obtained. Full length X1 encodes a protein of 738 amino acids with a predicted molecular mass of 78 kDa (PTPX1); full length X10 encodes a 652 amino acid protein with a predicted mass of 70 kDa (PTPX10). Both proteins belong to the non-transmembrane family of PTPases. PTPX1 and PTPX10 share segments of 97% identity in both the C-terminal catalytic domains and the N-terminal portions. In addition PTPX1 contains an additional 86 amino acid insert between the amino and carboxy terminal segments of the protein. The N-terminal half of both PTPX1 and PTPX10 are 28% identical to bovine cellular retinaldehyde binding protein, and 23% identical to sec14, a yeast phosphatidylinositol transferase. This similarity may indicate the potential for regulation of PTPX1 and PTPX10 by a lipid molecule. Sequence comparison and Northern blot analysis indicate that PTPX10 is likely to be the *Xenopus* homologue of the previously described human PTPMEG-2. PTPX1, on the other hand is a highly related, yet distinct, PTPase. Within the unique insert in PTPX1 are two Thr-Pro motifs which are consensus sites for phosphorylation by cdc2 and MAPK. The presence of these potential phosphorylation sites may indicate that although they are structurally similar, PTPX1 and PTPX10 may be differentially regulated. PTPX1 can be phosphorylated by $p42^{mapk}$ *in vitro*. To assess whether PTPX1 is regulated by phosphorylation *in vivo* we have expressed the enzyme in *Xenopus* oocytes. Expressed PTPX1 was recovered from oocytes in both soluble and membrane fractions. We are currently examining the biochemical properties of these two pools of PTPX1 to determine if subcellular localization and/or phosphorylation may regulate its function.

I 414 REGULATION OF RECEPTOR PROTEIN-TYROSINE PHOSPHATASE α ACTIVITY BY PHOSPHORYLATION Jeroen den Hertog and Tony Hunter
The Salk Institute, Molecular Biology and Virology Laboratory, 10010 North Torrey Pines Road, La Jolla CA 92037

Protein tyrosine phosphorylation is one of the main cellular signaling mechanisms, and plays a crucial role in cellular proliferation and differentiation. In recent years a number of protein-tyrosine phosphatases (PTPs) have been cloned. However, relatively little is known about their physiological role or the regulation of their enzymatic activity. Here we report that the enzymatic activity of a transmembrane PTP, Receptor Protein-Tyrosine Phosphatase α (RPTPα), is regulated by phosphorylation on both Ser as well as Tyr residues.

Stimulation of stably RPTPα-transfected P19 cells or transiently transfected 293 cells with phorbol ester leads to an increase in RPTPα PTPase-activity, as determined by an immunecomplex phosphatase assay, due to a two- to three-fold increase in substrate affinity. With similar kinetics RPTPα Ser-phosphorylation is transiently enhanced. The increase in Ser-phosphorylation is essential for the increase in activity, since in vitro dephosphorylation of RPTPα from phorbol-ester stimulated cells reduces its activity to pre-stimulation levels.

RPTPα, immunoprecipitated from NIH3T3 cells is phosphorylated on Ser-residues, and to a lesser extent on Tyr. Bacterially expressed PTPα (bPTPα) can be phosphorylated on Tyr in vitro, using immunoprecipitated Src. The major in vitro Tyr-phosphorylation site was mapped to Tyr789, 5 residues from the C-terminus of bPTPα. Tryptic phosphopeptide-mapping experiments of immunoprecipitated RPTPα and a mutant, containing a Tyr to Phe mutation at the position of the in vitro phosphorylation site, demonstrate that this Tyr-residue is phosphorylated in vivo. PTPα has auto-dephosphorylation activity in vitro, and conceivably also in vivo. Phosphatase assays with bPTPα, phosphorylated using ATPγS to prevent auto-dephosphorylation, indicate that phosphorylation of the Tyr-residue in the extreme C-terminus specifically inhibits PTPα-activity.

I 415 **MITOGEN-ACTIVATED PROTEIN KINASE IS INACTIVATED BY A TRANSCRIPTIONALLY REGULATED PROTEIN TYROSINE PHOSPHATASE IN RAT AORTIC SMOOTH MUSCLE CELLS**, Jennifer L. Duff and Bradford C. Berk, Department of Biochemistry, Emory University, Atlanta, GA 30322

Activation of mitogen-activated protein kinases (MAPK) by a protein phosphorylation cascade is an important signal transduction event initiated by a variety of stimuli. In rat aortic smooth muscle cells (RASM), angiotensin II (Ang II) rapidly activates MAPK (peak = 5 min). Inactivation is also rapid with return to baseline by 1 h. Recent in vitro studies suggest that 3CH134, a transcriptionally regulated protein tyrosine phosphatase (PTPase), specifically inactivates MAPK. To study the role of 3CH134 in Ang II regulation of MAPK in RASM, we examined 3CH134 mRNA and protein expression. Ang II (100 nM) rapidly induced 3CH134 mRNA by 10 min, with peak expression at 30 min (20-fold). Protein was detected in Ang II-stimulated RASM at 1 h by immunoprecipitation. Because 3CH134 is transcriptionally regulated, we studied the effects of actinomycin D on 3CH134 mRNA expression and MAPK inactivation. RASM were treated with actinomycin D (3 μg/ml for 30 min), then exposed to Ang II. Actinomycin D completely blocked 3CH134 mRNA expression. MAPK activity was assayed by an in-the-gel kinase assay (ITGKA). Myelin basic protein (MBP) was immobilized in a polyacrylamide gel, and following SDS-PAGE, kinases were renatured and detected by transfer of [γ^{32}P]-ATP to MBP. Ang II rapidly stimulated 5 MBP-kinases (42, 44, 70, 75 and 85 kDa) by 5 min, with return to baseline by 2 h. The 42 and 44 kDa proteins were identified as MAPK by Western blot analysis. Treatment with actinomycin D selectively prolonged the Ang II-stimulated activity of the 42 and 44 kDa proteins at 2 h and 4 h as measured by ITGKA. Actinomycin D treatment also prolonged the Ang II-stimulated phosphorylation of MAPK at 2 h and 4 h as measured by band shift on Western blot. Based on the time course for 3CH134 expression and the selective effect of actinomycin D we propose that 3CH134 is the PTPase responsible for inactivating MAPK in RASM in response to Ang II.

I 416 **FUNCTIONALITY OF PROLACTIN AND CHIMERIC PROLACTIN-ERYTHROPOIETIN RECEPTORS IN LYMPHOID CELLS.**

I. Dusanter, O. Muller, P. Mayeux, C. Lacombe, J. Djiane and S. Gisselbrecht. ICGM-INSERM U. 363, Hopital Cochin, Paris, FRANCE.

Prolactin (PRL) was originally identified as the main hormone controlling lactation. However recent studies suggested an immunomodulatory role for PRL in mammals. Moreover, PRL receptors (PRL-R) were shown to be members of the recently recognized cytokine receptor superfamily, which also includes the receptor for erythropoietin (Epo-R). To further investigate PRL actions on hematopoietic cells, mammary PRL-R cDNA was introduced in the IL-3 dependent pro-B lymphoid cell line BAF-3. In contrast to parental cells, PRL-R transfectants were maintained in long term culture in the presence of PRL instead of IL-3 without any loss of proliferative responsiveness. These cells expressed approximately 4000 R/cell which exhibited only low affinity for PRL (2 nM). PRL-R were characterized as 100,000KDa molecules by metabolic labeling and cross-linking experiments. A second (125I)oPRL-receptor complex, of 150,000 KDa, was also identified. In these cells, PRL induced the very rapid tyrosine-phosphorylation of its own receptor (in 1'), in addition to other receptor-associated molecules. Chimeric receptors cDNAs made of the PRL-R extracellular domain and the Epo-R intracellular domain were constructed and expressed in the same BAF-3 cells. The chimeras were active in sustaining the long-term growth of the transfected cells in the presence of PRL. Studies on their intracellular signalling pathways are currently under investigation. Therefore, cDNA coding for PRL-R can deliver a mitogenic signal in an hematopoietic cell. In addition,the PRL-R extracellular domain can complement the Epo-R intracellular domain resulting in a growth-promoting action in lymphoid cells.

I 417 THE TYPE II RECEPTORS FOR TGF-β OR ACTIVIN DETERMINE THE LIGAND SPECIFICITY OF A SINGLE TYPE I RECEPTOR, Reinhard Ebner, Ruey-Hwa Chen, Sean Lawler, Thomas F. Zioncheck, Alfredo R. Lopez, and Rik Derynck, Departments of Growth and Development, and Anatomy, University of California at San Francisco, San Francisco, CA 94143-0640, Department of Pharmacology, Genentech, 460 Point San Bruno Boulevard, South San Francisco, CA 94080.

Transforming growth factor-β (TGF-β) and the structurally related activin exert their biological activities primarily through interaction with type I and type II receptors. The type II receptors for TGF-β and activin are transmembrane serine-threonine kinases. A cloned type I TGF-β receptor is, based on its sequence, a serine-threonine kinase receptor as well and requires coexpression of the type II TGF-β receptor for ligand binding. We report that this type I receptor does not only bind TGF-β in the presence of the type II TGF-β receptor, but also has the ability to bind activin as well, when cotransfected with the type II activin receptor, and, in this context, has the characteristics of a type I activin receptor. Furthermore, this type I receptor physically associates with the type II receptor for TGF-β as well as for activin. Thus, the same receptor can act as a type I receptor for activin and TGF-β and possibly other ligands. The specificity of its ligand binding is conferred by the extracellular domain of the ligand-specific type II receptor.

I 418 PURIFICATION AND CHARACTERIZATION OF DELETIONAL MUTATIONS OF $pp60^{c\text{-}src}$ TYROSINE KINASE Byron Ellis, Pamela De Lacy, Debra Weigl, Indravadan Patel, G. Bruce Wisely, Kelly Lewis, Laurie Overton, Sue Cadwell, Thomas Kost, Christine Hoffman, George Barrett, Blain Knight, Ann Edison, Xinyi Huang, Daniel Kassel, Judd Berman, Marc Rodrigues and Michael Luther, Glaxo Research Institute, Research Triangle Park, NC 27709

The $pp60^{c\text{-}src}$ tyrosine kinase is associated with cell membranes via the myristylation of the N-terminal glycine. This reduces the solubility properties of $pp60^{c\text{-}src}$ making purification and characterization difficult. The solubility properties of $pp60^{c\text{-}src}$ can be improved by the use of detergent during the purification and/or the site-directed mutagenesis of the myristylation site. However, even upon these modifications $pp60^{c\text{-}src}$ has limited solubility and stability at high protein concentrations. In order to provide sufficient quantities of protein for biochemical and biophysical characterization studies we have designed various constructs of the c-src gene and expressed them in baculovirus infected sf9 cells. Purification methods using standard chromatographic techniques combined with affinity chromatography have been developed. The process yields protein which is >95% pure by SDS-PAGE and amino-acid sequence analysis. Isoforms of $pp60^{c\text{-}src}$ constructs have been identified by IEF and chromatofocusing. These isoforms represent differentially phosphorylated forms of $pp60^{c\text{-}src}$ by LC/MS. These isoforms can be resolved using weak anion exchange chromatography. The truncated constructs are enzymatically active, but exhibit altered affinities to c-src SH2 tyrosine phosphorylated target peptides when compared to the full length non-myristylated enzyme as determined by BIAcore. We discuss the expression, purification, and biochemical properties of these constructs and their affinities for c-src SH2 target peptides.

I 419 MUTATIONS IN THE SH2 AND KINASE DOMAINS OF V-*SRC* AFFECT INTERACTION WITH CELLULAR SUBSTRATES, Laura J. England, Eric C. Liebl, and G. Steven Martin, Department of Molecular and Cell Biology, 401 Barker Hall, University of California, Berkeley CA 94720

Host range mutants of v-*src* that transform chicken embryo fibroblasts (CEF) but are partially or completely transformation defective in Rat-2 cells were generated by linker-insertion mutagenesis (DeClue & Martin, J. Virol 63:542 [1989]). Mutations in the SH2 region of v-*src* affect the tyrosine phosphorylation of *ras*-GAP and the *ras*-GAP-associated protein p62 (Moran et al., Proc. Natl. Acad. Sci. USA 87:8622 [1990]). A mutation in the kinase domain (Y416 SRD), blocks the tyrosine phosphorylation of p36/annexin II (Liebl et al., J. Virol 66:4315 [1992]). These findings suggest that the host range phenotype may result from alterations in the substrate specificity of the v-*src* gene product or from other alterations that affect the tyrosine phosphorylation of cellular proteins.

To investigate these possibilities, we have examined the interactions of purified wild-type and mutant v-*src* gene products with the substrates enolase, p36/annexin II, and *ras*-GAP *in vitro*. The mutant v-*src* proteins have a K_m for acid-denatured enolase that is similar to that of wild-type v-*src*, but exhibit a decreased V_{max}. The kinase domain mutant has an increased K_m for p36. K_m determinations for *ras*-GAP are in progress.

We are also investigating whether the non-catalytic functions of v-*src* can be supplied *in trans* to rescue the transformation defects of the v-*src* SH2 mutants. In Rat-2 cells expressing v-*src* SH2 mutants, co-expression of a v-*src* construct (N2) lacking the kinase domain enhances transformation but does not increase *ras*-GAP phosphorylation. These findings suggest that the SH2 region of *src* may play a role in the assembly of signaling complexes, the targeting of phosphorylated substrates or some other function that can be supplied *in trans*.

I 420 PERVANADATE MIMICS THE EFFECTS OF IL-2 IN HUMAN T CELLS: EVIDENCE FOR THE ACTIVATION OF TWO DISTINCT TYROSINE KINASE PATHWAYS BY IL-2, Gerald A. Evans, Rebecca Erwin, O. M. Zack Howard, Robert A. Kirken and William L. Farrar, Biological Carcinogenesis and Development Program, Program Resources Inc./DynCorp and the Laboratory of Molecular Immunoregulation, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702

Pervanadate has been shown to be a potent inhibitor of phosphotyrosine phosphatase activity and in hematopoietic systems has been shown to mimic some events associated with engagement of the T cell receptor. In this study we determined to what extent pervanadate can mimic IL-2 stimulated events in human T cells. In YT cells (a NK-like T cell line) and in lectin activated T cells, pervanadate induces proliferation as well as interferon gamma production without inducing production of IL-2. The production of interferon gamma by pervanadate is synergistic with that stimulated by IL-2. Pervanadate also induces a rapid increase in tyrosine phosphorylation, increases the activity of the src-family kinases lck and fyn, and induces increased levels of fos and jun transcript. When compared to IL-2, pervanadate induces tyrosine phosphorylation of overlapping substrates with the exception of a receptor associated 116 kDa phosphoprotein. Further analysis of receptor associated tyrosine kinase activity shows that pervanadate unlike IL-2 does not affect IL-2 receptor tyrosine kinase activity. These results suggest that pervanadate can mimic the effects of IL-2 by bypassing receptor activation and activating downstream tyrosine kinases. Of the dominant tyrosine kinase substrates phosphorylated in response to IL-2, pp116 is strictly dependent on IL-2 and can not be rapidly induced by pervanadate. This supports the contention that IL-2 activates a receptor associated tyrosine kinase pathway involving p116 phosphorylation which is distinct from the downstream pathways involving members of the src-family of tyrosine kinases.

I 421 Critical Tyrosine Residues Regulate the Enzymatic and Biological Activity of the Raf-1 Kinase. John R. Fabian[1], Ira Daar[2], and Deborah K. Morrison[1]. [1]ABL-Basic Research Program and [2] Laboratory of Leukocyte Biology, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702

The serine/threonine kinase activity of the Raf-1 proto-oncogene is stimulated by the activation of many tyrosine kinases, including growth factor receptors and pp60v-src. Recent studies of growth factor signal transduction pathways demonstrate that Raf-1 functions downstream of activated tyrosine kinases and p21ras and upstream of mitogen activated protein kinase (MAPK). However, coexpression of both activated tyrosine kinases and p21ras are required for maximal activation of Raf-1 in the baculovirus/Sf9 expression system. In this study, we investigated the role of tyrosine kinases and tyrosine phosphorylation in the regulation of Raf-1 activity. Using the baculovirus/Sf9 expression system we identified Tyr340 and Tyr341 as the major tyrosine phosphorylation sites of Raf-1 when coexpressed with activated tyrosine kinases. Introduction of a negatively charged residue that may mimic the effect of phosphorylation at these sites activated the catalytic activity of Raf-1 and generated proteins that could transform Balb/3T3 cells and induce the meiotic maturation of Xenopus oocytes. In contrast, substitution of non-charged residues that were unable to be phosphorylated produced a protein that could not be enzymatically activated by tyrosine kinases and that could block the meiotic maturation of oocytes induced by components of the receptor tyrosine kinase pathway. These findings demonstrate that mutation of the tyrosine phosphorylation sites can dramatically alter the function of Raf-1. In addition, this is the first report that a transforming Raf-1 protein can be generated by a single amino acid substitution.

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I 422 AN ANALYSIS OF AXONAL RECEPTOR-LINKED PROTEIN TYROSINE PHOSPHATASES IN DROSOPHILA, Sarah J. Fashena, Shin-Shay Tian, and Kai Zinn, Division of Biology 216-76, Caltech, Pasadena, CA 91125.

We have previously identified three receptor-linked protein tyrosine phosphatases (R-PTPs) that are expressed predominantly on CNS axons during *Drosophila* embryo development. In an effort to elucidate how the R-PTPs DPTP99A and DPTP10D contribute to the development of the nervous system, we are searching for their intracellular substrates and extracellular ligand(s).

To identify other components of the signaling pathways in which the R-PTPs act, we have cloned a putative substrate of DPTP10D. A 150 kD transmembrane protein (denoted p150) that binds to the cytoplasmic domain of DPTP10D has been partially sequenced and p150 cDNAs have been isolated. Significantly, phosphorylated p150 is a preferred substrate for DPTP10D activity in vitro. The extracellular domain of p150 contains 'leucine-rich repeats' homologous to those of the Drosophila adhesion molecules chaoptin and connectin. The short cytoplasmic domain contains three tyrosine residues in a sequence context similar to those of 'trigger' signal motifs of the T cell receptor-associated CD3 and ζ chains. When phosphorylated, these trigger motifs bind to SH2 domain-containing signaling proteins. The p150 gene is located at 58D on the polytene chromosomes; p150 mRNA is primarily expressed in patches of epithelium at or near muscle attachment sites. We are currently attempting to show that p150 and DPTP10D are associated in vivo.

To create probes with which to isolate potential R-PTP ligands and/or proteins that complex with R-PTPs within the same membrane, we have made fusion proteins which include all or subportions of the DPTP99A and DPTP10D extracellular regions linked to a human Fc domain. The fusion proteins are denoted Rg99AFN3/TM, which includes all three of the DPTP99A fibronectin type III (FN) domains, and Rg10DFN4/5, which includes the four most distal DPTP10D FN domains. Rg10DFN4/5 immunoprecipitates a 48 kD protein in extracts derived from metabolically labeled *Drosophila* primary neuronal cultures and Schneider (S2) cells. The 48 kD protein can be isolated from total cell lysates or from S2 cell media. Rg99AFN3/TM immunoprecipitates a 58 kD protein in extracts from labeled neuronal and S2 cells. The 58 kD protein is localized to the plasma membrane fraction in membrane preparations. We are currently investigating whether the 48- and 58-kD proteins are expressed on the cell surface and plan to purify these proteins in sufficient quantities to obtain sequence information.

I 423 PHOSPHORYLATION OF THE PROTEIN TYROSINE PHOSPHATASE PTP1B BY MULTIPLE SERINE/THREONINE KINASES, Andrew J. Flint and Nicholas K. Tonks, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724

The phosphorylation of proteins on tyrosine residues is a reversible covalent modification that is linked to control of cell growth and differentiation. The protein tyrosine phosphatases (PTPases) encompass a diverse group of enzymes which in concert with the protein tyrosine kinases control the levels of cellular phosphotyrosine. PTPases typically contain non-catalytic segments suggestive of targetting or regulatory functions. PTP1B consists of an amino-terminal 30 kDa catalytic domain connected by a stretch of 115 predominantly hydrophilic amino acids to a short hydrophobic sequence at the extreme carboxy-terminus, that anchors PTP1B in the cytoplasmic face of the endoplasmic reticulum membrane. The hydrophilic segment is phosphorylated by multiple serine/threonine kinases and appears to play an additional regulatory role. In asynchronously growing HeLa cells, PTP1B is phosphorylated on three tryptic peptides. Phorbol ester treatment enhances the phosphorylation of one of these peptides via PKC phosphorylation of Ser378. In mitotically arrested HeLa cells dramatic changes in the sites of phosphorylation of PTP1B are observed, concomitant with a ~30% decrease in its enzymatic activity. Under these conditions Ser378 is desphosphorylated while at least two new phosphopeptides are detected, one resulting from $p34^{cdc2}$ phosphorylation of Ser386 while Ser352 is phosphorylated by an as yet unidentified kinase. Recent data suggest that the Ser352 kinase may be a MAP kinase-like activity; in cotransfection experiments with positive and negative regulators of MAPK activity the phosphorylation of Ser352 is correspondingly increased or decreased. Transfection of 293 cells with mutants at each of the identified phosphorylation sites has enabled analysis of interactions between the different phosphorylation events. These data suggest that phosphorylation of Ser352 triggers dephosphorylation of the PKC phosphorylation site Ser378 while phosphorylation by $p34^{cdc2}$ occurs independently of the phosphorylation state of either of these two other sites. These results highlight the complex nature of the interplay between pathways involving serine/threonine kinases and protein tyrosine dephosphorylation.

I 424 *SPRK*: AN SH3 DOMAIN-CONTAINING PROLINE-RICH KINASE WITH SERINE/THREONINE KINASE ACTIVITY, Kathleen A. Gallo, Melanie R. Mark, and Paul J. Godowski, Department of Cell Genetics, Genentech, South San Francisco, CA 94080

Protein kinases play important roles in the growth and differentiation of cells. We have isolated cDNA clones from the human megakaryocytic cell line CMK11-5 that encode a novel protein kinase, which we call *sprk*. The gene sequence predicts an 847 amino acid protein kinase with a unique domain structure. An amino terminal glycine-rich region is followed by an SH3 domain and a kinase domain that is similar to both tyrosine and serine/threonine kinases. Adjacent to the kinase domain are two closely-spaced leucine/isoleucine "zipper" motifs, and a stretch of basic amino acids that resembles a karyophilic nuclear localization signal. The *C*-terminal half of *sprk* is basic, and proline accounts for 24% of the *C*-terminal 216 amino acids.

The *sprk* gene is widely expressed as a 4 kb transcript in adult and fetal tissues. Transfection of 293 cells with a vector encoding an epitope-tagged *sprk* results in the expression of a 94 kDa protein which fractionates primarily with cytoplasmic extracts. The epitope-tagged *sprk* becomes phosphorylated on serine and threonine residues in an *in vitro* kinase assay, whereas *sprk* variants with point mutations in the predicted ATP binding site fail to become phosphorylated. These data indicate that *sprk* has serine/threonine kinase activity. The SH3 domain of *sprk* is interrupted by a unique five amino acid "insert" whose location in the SH3 consensus sequence is the same as that of the "inserts" found in the SH3 domains of neural src and of the p85 subunit of phosphatidylinositol 3-kinase. Since there are suggestions that this region may be involved in ligand specificity, we are taking both a biochemical and expression cloning approach towards identifying proteins that interact with the SH3 domain of this kinase. We have also undertaken immunofluoresence studies to determine the subcellular localization of *sprk*, and are presently investigating the effects of mutations in various domains of *sprk* on its localization and kinase activity.

I 425 PTP-PEST: A PROTEIN TYROSINE PHOSPHATASE REGULATED BY REVERSIBLE PHOSPHORYLATION, Andrew J. Garton & Nicholas K. Tonks, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

The protein tyrosine phosphatases (PTPs) are a structurally diverse family of enzymes comprising both transmembrane, receptor-like enzymes and non-transmembrane forms. The catalytic domains of these enzymes are generally very highly conserved, whereas the structures of the extra-catalytic segments are extremely divergent, offering the potential for differential regulation and/or targetting of each member of the PTP family. Tyrosine phosphorylation of proteins is an essential step in a wide variety of signal transduction pathways involved in cell growth, division and differentiation; this fact together with the observed high specific activity of the PTPs when assayed *in vitro* suggests a requirement for tight regulation of PTP activity within the cell to permit these processes to occur in a controlled fashion. We are studying these control mechanisms, with particular emphasis on the potential involvement of Ser/Thr phosphorylation/dephosphorylation of PTPs in their regulation. Such mechanisms offer an attractive means whereby signal transduction pathways involving Ser/Thr phosphorylation can potentially interact with, and modulate events controlled by protein tyrosine phosphorylation.

PTP-PEST is an 88 kDa non-transmembrane human PTP recently cloned from a HeLa cell cDNA library. The protein consists of a 28 kDa catalytic domain, flanked by 57 amino acid residues at the N-terminus, and a 53.5 kDa C-terminal segment which is highly enriched in Pro, Glu/Asp, and Ser/Thr residues, a feature that often predicts proteins with short biological half-lives. The protein is expressed in a wide variety of cell lines including A204 rhabdomyosarcoma, HeLa, 293, and PC 12 cells. We have expressed PTP-PEST in Sf9 cells using recombinant baculovirus, and purified the enzyme essentially to homogeneity. The purified enzyme has been found to be phosphorylated at multiple sites by several protein Ser/Thr kinases, we have identified two of these sites. Phosphorylation of PTP-PEST at one of these sites has profound effects on enzyme activity, resulting in an up to 70% reduction in the activity of the enzyme. Furthermore, this site is also phosphorylated in intact HeLa cells, suggesting that PTP-PEST is an example of a protein tyrosine phosphatase whose activity is regulated *in vivo* by Ser/Thr phosphorylation. The nature of the kinases involved and the identification of the phosphorylation sites will be presented.

I 426 PROBING SIGNAL TRANSDUCTION PATHWAYS: HUMAN src SH2 DOMAIN AND ITS PARTNERS, Tona Gilmer, Marc Rodriguez, Michael Green, Craig Wagner, Steve Jordan, Paul Charifson, Deirdre Luttrell, Amanda Lee, Renae Crosby, Krystal Jung, Tim Lansing, Annie Hassell, Derril Willard, Mike Luther, Judd Berman, Departments of Cell Biology, Medicinal Chemistry, Structural and Biophysical Chemistry, Glaxo Research Institute, Research Triangle Park, NC 27709.

There is growing evidence that SH2 domains of nonreceptor tyrosine kinases are critical to the function of these proteins in signal transduction. We have examined the protein-protein interactions of the src SH2 domain by affinity precipitation from human tumor-derived cell lines, *in vitro* binding assays, and X-ray crystallographic methods. EGFR and HER2/neu were identified as binding to the src SH3-SH2 domains in human breast carcinoma cell lines which overexpress these receptor tyrosine kinases, and SHC binding was detected in colon carcinoma cell lines. Over 100 peptides have been tested to probe the structure activity relationship for inhibitors of src SH2 interactions. Inhibitors of src SH3-SH2/EGFR interaction have been identified using a competitive ELISA. These peptides correspond to sequences surrounding tyrosine residues from src, EGFR, FAK (focal adhesion kinase), and the Y*EEI sequence reported by Songyang et al (Cell, 1993). X-ray crystallography and modeling studies using the src SH2 domain in complex with phosphotyrosyl peptides have defined further the ligand requirements for binding as well as residues within the src SH2 domain that are involved in binding.

I 427 UNIQUE AND REDUNDANT SIGNAL TRANSDUCTION ELEMENTS IN THE IL-2 RECEPTOR β CHAIN, Mark A. Goldsmith, Weiduan Xu, M. Catherine Amaral, Elizabeth Kuczek, and Warner C. Greene, Gladstone Institute of Virology and Immunology, University of California, San Francisco, San Francisco, CA 94141-9100.

Binding of IL-2 to the IL-2 receptor (IL-2R) triggers a series of events that result in lymphocyte proliferation and/or differentiation. The β and γ subunits of IL-2R are members of the cytokine receptor superfamily that appear to mediate the early steps in this signal transduction process. To facilitate identification of intracellular domains that contribute to these events, we have developed a novel transient assay of signal transduction. This method measures proliferation of a growth factor-dependent cell line following transient transfection of expression vectors encoding wild type or mutant receptor chains. Both IL-2R and the erythropoietin receptor support large proliferative responses in this protocol, providing an assay with broad dynamic range and high sensitivity to impairment of receptor function. We have combined this assay with cassette mutagenesis to define domains and specific residues within the IL-2Rβ cytoplasmic region that contribute to signal transduction. We found that replacement of the transmembrane domain with that of the CD4 molecule had no impact on signalling competence. Internal deletion of either the "Box 1" or "Box 2" proximal cytokine receptor homology segments significantly impaired receptor function. In contrast, deletion of the 119-amino acid segment immediately downstream of Box 2 had no effect on receptor function; this dispensable region encompasses both the previously-described "A" domain and an additional 50 downstream residues ("B" domain). Deletion of the C-terminal 94 residues of this chain ("C" segment") partially impaired signalling, while deletion of both "B" and "C" regions markedly diminished receptor function. Systematic substitution analysis of the proximal receptor region revealed several residues within and between Box 1 and Box 2 that appear to contribute substantially to receptor competence. These studies thus define the functional architecture of the intracellular region of IL-2Rβ; this structure includes a fully dispensable segment comprising the central one-third of the cytoplasmic tail, as well as both proximal and distal elements that significantly influence signalling function. Both unique and functionally redundant elements are identifiable.

I 428 IDENTIFICATION OF P-TYR PHOSPHOPROTEINS AND AN EGF RECEPTOR HOMOLOG IN TRYPANOSOMA CRUZI.
Mary Isabel Gonzatti[1], Andreas Batzer[2], José Bubis[1] and Joseph Schlessinger[2.1] Universidad Simón Bolívar, Depto. Biología Celular, Caracas, Venezuela. [2] New York University, Dept. of Pharmacology, U.S.A.
Trypanosoma cruzi, a flagellated protozoan and the etiologic agent of Chagas' Disease, undergoes dramatic morphological and physiological changes throughout its life cycle in the insect vector and its infected mammalian host. The study of signal transduction pathways in this parasite should help unravel the mechanisms involved in its adaptation to an obligate parasitic life style. Three different anti-P-tyrosine antibodies were used to examine T.cruzi homogenates by Western blot analysis. We found qualitative and quantitative differences in the pattern of P-Tyr phosphoproteins when the various stages of the parasite were compared. Epimastigotes (Epi), the non-infective, proliferative forms, differentiate into Trypomastigotes (T), a non-proliferative, infective stage and finally to Spheromastigotes (S), which are rounded, non-flagellated and highly infective forms of the parasite. The three different anti-P-tyr antibodies detected four proteins with relative molecular weights of 160,000 (p160); 18,000 (p18); 17,000 (p17) and 12,000 (p12), which were present in all stages of the parasite. Three other proteins, p75, p70 and p26 were only detected in the infective T and S forms and p90 was absent from the T stage. Western blot analyses of T.cruzi Epi lysates, using an antibody against the human EGF receptor kinase domain, revealed a p160 band that was enriched in the membrane fraction. This p160 was immunoprecipitated by the anti-EGFR antibody and phosphorylated in an in vitro kinase assay. Thus, the p160 identified by three different anti-P-tyr antibodies appears to be a T. cruzi EGF receptor homolog.

I 429 A MONOCLONAL ANTIBODY AGAINST YEAST POLYPEPTIDE-DEPENDENT PROTEIN KINASE (PK-P): ANALYSIS OF ENZYME ACTIVATION AND CELLULAR LOCALIZATION IN YEAST AND RAT FIBROBLASTS,
John Gordon, Walter Kaczmarczyk and Gerald A. Evans, Genetics and Developmental Biology Program, West Virginia University, Morgantown WV, 26506 and Biological Carcinogenesis and Development Program, Program Resources Inc./DynCorp, National Cancer Institute, FCRDC, Frederick, MD 21702
Polypeptide-dependent protein kinase (PK-P) is a serine/threonine kinase of 75 kDa (41 kDa catalytic and 35 kDa non-catalytic subunit) which is similar to casein kinase II and was originally isolated from human placenta. PK-P has been shown to be induced by mitogenic growth factors and to regulate the activity of the EGF receptor. Because of the low antigenicity of PK-P, monoclonal antibodies were produced using *in vitro* immunization of naive Balb/c mouse splenocytes followed by fusion to Ag8.653 myeloma cells. Analysis of antibody producing clones revealed a clone, denoted 2B3, that produced antibody which recognized the native form of the enzyme. Analysis of a partially purified preparation of PK-P revealed four distinct bands of 92, 75, 63 and 45 Kda which reacted with 2B3 following non-denaturing PAGE and immunoblotting. Using a gel overlay assay we show that the 63 and 45 kDa proteins are autophosphorylated and sensitive to heparin. Further analysis shows that both p45 and p63 are autophosphorylated, stimulated by histone, and inhibited by RNA, heparin and dinitrophenol. All are characteristics of purified PK-P and suggest that the 63 kDa protein represents a previously unidentified catalytic form of PK-P. Immunolocalization shows that PK-P and immunologically similar proteins are associated with yeast cytoplasmic membranes and with cytoplasmic and nuclear membranes in NRK-49f rat firbroblasts.

I 430 IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF SH3 DOMAIN-BINDING PROTEINS,
Ivan Gout*, Ritu Dhand*, Ian D. Hiles*, Michael J. Fry*, George Panayotou*, Pamela Das*, Oanh Truong*, Nicholas F. Totty*, Justin Hsuan*, and Michael D. Waterfield*†, *Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT, UK.
Structural and functional studies of SH2 and SH3 domains have identified several signal complex molecules which couple receptor tyrosine kinases to potential second messenger systems including Ras activation. Here we describe the use of affinity purification methods to search bovine brain extracts for SH3 domain-binding proteins. Using this approach we have purified and obtained partial sequences for several proteins (P55, P70, P100, P150) which selectively associated with different SH3 domains. One of these molecules (P100) was found to be the previously identified GTPase dynamin. This protein showed selective binding to a panel of 15 SH3 domains which was mediated by a C-terminal proline-rich sequence. Proline-rich peptides derived from dynamin, 3BP1, and SOS1 exhibited differential binding towards different SH3 domains. A subset of SH3 domains was also shown to stimulate significantly the intrinsic GTPase activity of dynamin. This observation is the first indication that these domains are not only involved in protein-protein interaction, but that they can also regulate the function of the associated molecules.

I 431 TNF RECEPTOR TR80 IS A SENSITIZER OF TR60 MEDIATED CYTOTOXICITY,
Matthias Grell, Beate Maxeiner, Gudrun Zimmermann, Klaus Pfizenmaier and Peter Scheurich, Institute of Cell Biology and Immunology, University of Stuttgart, 70569 Stuttgart, Germany
Tumor necrosis factor mediated tumor cell lysis is hampered by a constitutive or TNF induced resistance to TNF and, *in vivo*, severe systemic side effects. We here show that a simultaneous treatment with TNF and a noncompetitive TR80 specific mAb renders *a priori* TNF resistant tumor cells highly sensitive to the cytotoxic action of TNF. These data provide new insights into the functional role of TR80 TNF receptors.

So far, most data have shown that TR60 is the principle mediator of TNF induced cytotoxicity, whereas the role of TR80 is still controversially discussed. To investigate the contribution of TR80 in different cellular TNF responses we have employed receptor specific antibodies, that do not compete for ligand binding, but are capable to modulate cellular TNF effects. Whereas incubation of Colo 205 cells with high concentrations of TNF or an agonistic TR60 specific antibody alone did not induce cytotoxicity, treatment of cells with a TR80 specific mAb (80M2) in the presence of TNF resulted in a strong cellular response. No effects were observed when the cells were treated with 80M2 alone or in the presence of the TR60 specific agonist. These data indicate that in Colo 205 cells I. TNF signalling *via* TR80 depends on TNF binding to TR80 and a secondary signal provided by the antibody and II. that this signal is *per se* not sufficient to induce cytolysis but requires simultaneous activation of TR60 receptors.

Similarly, TNF resistant subclones of the cell line KYM-1 could be also reverted to a sensitive phenotype by cotreatment with TNF and 80M2. These findings imply a novel role of TR80 as a sensitizer of TR60 mediated TNF effects. We suggest that this antibody mediated enhancement of TNF's action *via* TR80 could represent a different quality of signal transduction. A physiological (co)activator of TR80 could be the membrane form of TNF or an yet unidentified TR80 associated protein.

I 432 AN α-SUBUNIT MUTATION IN THE INSULIN RECEPTOR CONSTITUTIVELY ACTIVATES RECEPTOR TYROSINE KINASE, WITH NO EFFECT ON CELL GROWTH OR KEY SIGNALLING ENZYMES.

K. Grønskov[1], B. Ursø[1], L.M. Graves[2], E. Van Obberghen[3] and P. De Meyts[1], [1]Hagedorn Research Institute, Niels Steensensvej 6, DK 2820 Gentofte, Denmark, [2]Dept. of Pharm., Univ. of Washington, Seattle, WA 98195, [3]INSERM U. 145, Faculte de Médecine, Nice, France.

Insulin receptors from a patient with heritable insulin resistance were reported to have constitutively increased autophosphorylation and tyrosine kinase activity, and to have lost high affinity insulin binding (Longo N., et al BBRC, 167, 1229, 1990). Glucose transport has been reported to be constitutively activated in the patient's fibroblasts and transfected cells (Longo N., et al PNAS, 90, 60, 1993). This was due to a homozygous mutation in the insulin receptor alpha-subunit, substituting Arg86 for Pro (R86P) (Longo N., et al Clin. Res. 40, 2, 239A, 1992).

We created the R86P insulin receptor by site-directed mutagenesis and stably transfected it into BHK cells (BHK-R86P), as well as the wild type insulin receptor (BHK-HIR) and the vector alone (BHK-NEO). The receptor showed no insulin binding above background when expressed in cells or when purified with WGA (Grønskov K., et al. BBRC, 192, 905, 1993). Immunocytochemistry showed an intracellular localization of the receptor. Western blotting with an anti-insulin receptor antibody indicated that the R86P receptor is not processed properly. Basal autophosphorylation of the receptor was constitutively increased as was tyrosine kinase activity towards a synthetic substrate (Grønskov K., et al. BBRC, 192, 905, 1993).

We have further investigated the R86P receptor with respect to growth, mitogen activated protein kinase (MAPK) activity and phosphoinositol-3-kinase (PI3-K) activity. BHK-R86P does not show any differences in growth rates compared to BHK-NEO and BHK-HIR when grown in media with 10 % fetal calf serum (FCS), 0.1 % FCS or 0.1 % FCS + 1 ug/ml insulin. MAPK activity was measured both in cell extracts and in immunoprecipitates with anti-MAPK antibody towards myelin basic protein, and was found not to be increased in BHK-R86P compared to BHK-NEO. The PI3-K activity was measured in immunoprecipitates with anti-p85 antibody towards PtdIns, and analysed on thin layer chromatography. The PI3-K activity was not increased in BHK-R86P compared to BHK-NEO. These results show that a constitutively activated insulin receptor tyrosine kinase which is not processed properly is not sufficient for triggering the mitogenic pathway of insulin.

I 433 FISSION YEAST PROTEIN TYROSINE PHOSPHATASES pyp1 AND pyp2 FUNCTION IN THE ABSENCE OF CATALYTIC ACTIVITY,

Gerhard Hannig[1,2], Sabine Ottilie[1,3] and Raymond L.Erikson[1], 1-Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138, 2-Beth Israel Hospital and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115, 3-Department of Molecular Biology MB-3, The Scripps Research Institute, La Jolla, CA 92037

During the 1980s, tyrosine phosphorylation was recognized as a key regulator of a wide range of cellular processes such as proliferation and differentiation. The importance of tyrosine dephosphorylation was less clear, primarily due to the absence of characterized phosphatases specific to tyrosine dephosphorylation (PTPases). Yeast genetics has clearly established the importance of PTPases in cell cycle control. In the fission yeast *Schizosaccharomyces pombe*, the activation of the $p34^{cdc2}$/cyclin B complex (which triggers the transition from G_2 into mitosis) is dependent on the dephosphorylation on tyrosine-15 of $p34^{cdc2}$. This dephosphorylation is catalyzed by the cdc25 phosphatase, probably in cooperation with another PTPase designed pyp3. Yet the importance of PTPases in the regulation of the fission yeast cell cycle is not restricted to an activator function, as two other PTPases (pyp1 and pyp2) were found to act as negative regulators of mitosis upstream of the $wee1^+/mik1^+$ pathway. Because of this novel and unexpected finding, we are interested in further characterizing both enzymes. Here we present evidence that catalytically inactive versions of pyp1 and pyp2 display biological activity similar to their wild-type counterparts, and discuss possible implications of this finding.

I 434 THE IMPORTANCE OF THE CONSERVED HELIX MOTIF THAT INTERACTS WITH THE CATALYTIC CORE OF cAMP-DEPENDENT PROTEIN KINASE

Friedrich W. Herberg and Susan S. Taylor, Department of Chemistry, Univ. of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0654, USA

cAMP-dependent protein kinase (cAPK) is one of best characterized members in the diverse family of protein kinases, although in their catalytic core region these enzymes are highly conserved. The inactive tetrameric holoenzyme contains two regulatory (R) and two catalytic (C) subunits and this complex is dissociated in response to nanomolar molar concentrations of the second messenger, cAMP. Besides the highly conserved catalytic core (residues 42 to 294) a conserved helix motif (A-helix) is found at the N-terminus of the C-subunit of cAPK. A predominant feature of this helix is a highly conserved Trp at the C-terminus of the helix. This Trp fills a hydrophobic pocket that lies precisely between the two lobes. In addition to the A-helix, residues 1-14 encode a myristylation motif. In the mammalian enzyme the N-terminus is myristylated, and the myristate is anchored to a hydrophobic pocket on the surface of the large lobe. This A-helix motif is predicted to be important for the structural integrity of the kinase core in cAPK while in other protein kinases it could also serve as a linker to other domains (1, 2). In order to understand the importance of the A-helix for interaction with the R-subunit as well as its importance for the stability of the protein, two deletion mutants were made in the N-terminus of the mouse C-subunit and overexpressed in *E.coli* . The first mutation (deletion 1-14) lacked the myristylation motif while the deletion 1-39 lacks the A-helix as well. In addition, Trp 30 was replaced with both a Tyr and Ala.

Both the 1-14 deletion mutant and the two point mutations were catalytically indistinguishable from wild-type C-subunit, however, all three were significantly more labile against heat denaturation than the wild-type protein. All three mutants also formed stable holoenzymes with the RI-subunit and these were more stable than the wild-type holoenzymes. The deletion 1-39 mutation, was purified as a fusion protein with glutathion-S-transferase. This fusion protein was catalytically active and can be inhibited by the R-subunit.

1. Veron, M., Radzio-Andzelm, E., Tsigelny, I., Ten Eyck, L. F. and Taylor, S. S. (In press 1993) *Proc. Natl. Acad. Sci. USA* .

2. Knighton, D. R., Zheng, J., Ten Eyck, L. F., Ashford, V. A., Xuong, N.-h., Taylor, S. S. and Sowadski, J. M. (1991) *Science* **253**, 407-414.

I 435 REGULATION OF THE HUMAN CELL CYCLE BY cdc25 PHOSPHATASES

Ingrid Hoffmann, Paul R.Clarke, *Giulio Draetta and Eric Karsenti, Cell Biology Programme, European Molecular Biology Laboratory, Meyerhofstr.1, 69117 Heidelberg, Germany and *Mitotix, 1 Kendall Sq., Cambridge, MA 02139

Dephosphorylation and activation of the cdc2/cyclinB kinase is a critical step in the initiation of entry into mitosis. Cdc25 phosphatases of the C-type are responsible for the dephosphorylation of cdc2 on Thr-14 and Tyr-15 residues. Our studies demonstrated that cdc25-C phosphatase is activated directly by phosphorylation in mitotic HeLa cells. This activation is due to phosphorylation by the cdc2/cyclinB kinase itself. Thus, phosphorylation of cdc25-C is probably sufficient to switch on an autocatalytic positive feed-back loop where active cdc2/cyclinB phosphorylates and activates more cdc25-C, resulting in the dramatic activation of cdc2/cyclinB and entry into mitosis.

We are currently studying the function and regulation of another member of the human cdc25 phosphatase family, cdc25-A. Cdc25-A undergoes phosphorylation and activation at earlier stages in the cell cycle than the cdc25-C phosphatase. Furthermore, microinjection of antibodies against cdc25-A into human fibroblasts inhibit the entry of the cell into S-phase. Since Thr-14 and Tyr-15 residues are conserved within the family of cyclin-dependent kinases, the cdc25-A phosphatase could play a critical role in the regulation of the G1/S transiton of the cell-cycle.

I 436 ANALYSIS OF MICE CARRYING A NULL MUTATION IN THE RIβ REGULATORY SUBUNIT OF CAMP-DEPENDENT PROTEIN KINASE, Rejean L. Idzerda, Eugene P. Brandon, Kirstin A. Gerhold, and G. Stanley McKnight, Department of Pharmacology, University of Washington School of Medicine, Seattle WA 98195

The RIβ regulatory subunit of cAMP-dependent protein kinase (cAPK) is predominantly expressed in neural tissues, and is found at lower levels in adrenal and gonadal cells. To determine whether this regulatory subunit isoform plays an essential role in development and function of these tissues, we have generated $RI\beta^{-/-}$ null mice by gene disruption. The RIβ gene was targeted by homologous recombination in ES cells derived in our own laboratory, as well as in the D3 ES cell line (derived by Doetschman and Kemler). Chimeras made from each disrupted cell line have passed the null mutation through the germ line generating heterozygotes, which have been crossed to obtain mice homozygous for the mutation. The $RI\beta^{-/-}$ mice are viable, survive to adulthood, and exhibit no gross abnormalities in appearance or behavior. The mice are currently being mated to assess fertility. To determine whether tissues that normally express RIβ can develop properly in the absence of RIβ, the $RI\beta^{-/-}$ mice are being crossed with a transgenic mouse line expressing RIβ-lac (the lacZ gene driven by the mouse RIβ promoter). β-gal staining will be used to follow the fate of RIβ expressing tissues in the resulting $RI\beta^{-/-}$ X RIβ-lac mice. The cAPK system appears to play a role in neuronal synaptic plasticity and in complex behaviors such as learning and memory. The RIβ regulatory subunit, which is expressed in hippocampus, cortex, and other neural tissues, may be important in neural function. Thus, both electrophysiological and behavioral studies are being used to evaluate the neural function of the $RI\beta^{-/-}$ mice.

I 437 CONSEQUENCES OF SELECTIVE INHIBITION OF HER2 PROTEIN TYROSINE KINASE BY GELDANAMYCIN IN HER2-OVEREXPRESSING MOUSE FIBROBLASTS. Janicot, M., Boiziau, J., Parker, F., Le Roux, M., Bonnevaux, H., Dugué A. & Tocqué, B. Rhône-Poulenc Rorer, Molecular Oncology Dept, Vitry s/Seine, France.

The *HER2* gene, the human homolog of the rat protooncogene *neu*, codes for a 185-kD transmembrane glycoprotein with extensive sequence and structure similarities with the human epidermal growth factor receptor (EGFR). HER2 protein possesses intrinsic tyrosine-specific kinase activity able to catalyze autophosphorylation and to mediate phosphorylation of endogenous substrates. Overexpression of HER2 has been implicated in morphological transformation and tumorigenesis. For instance, the unaltered *HER2* gene has been found amplified in various human tumors including adenocarcinomas of the breast, ovary, lung, stomach, colon, kidney, and lung carcinomas. Since implication of the solely HER2 tyrosine kinase activity in tumorigenesis is still under investigations, it was of extreme interest to assess the role of this kinase in oncogenesis and to investigate the significance of the correlation between overexpression of this gene and cell transformation. In this general issue, finding of specific tyrosine kinase inhibitors has been widely documented. In this study, we investigated the effect of geldanamycin (GDM), an antibiotic of the benzoquinoid ansamycin group, on HER2 protein tyrosine kinase. GDM has been previously shown to reverse the oncogenic phenotype of v-src-transformed cell lines by inducing irreversible loss of v-src tyrosine kinase activity. We showed that GDM is a highly potent inhibitor of HER2 autophosphorylation in intact NIH3T3 cells overexpressing *HER2* (IC_{50} at 50-100 nM), without any effect on *in vitro* autophosphorylation of immunopurified HER2. Total but reversible inhibition of HER2 autophosphorylation is achieved 2-3 h after addition of the compound. Under these conditions, upon incubation of NIH3T3 cell lines overexpressing various tyrosine kinases with GDM, we could observe selective inhibition of HER2 autophosphorylation as compared to EGFR-, v-src- and insulin receptor β subunit-associated tyrosine kinases. Although inactive on EGFR-associated tyrosine kinase, GDM inhibited EGF-stimulated DNA synthesis in intact cells with an IC_{50} of 400 nM. Attempts to elucidate the mechanism of inhibition of HER2 tyrosine kinase by GDM will be discussed, in particular, whether this compound could also interfere with the binding of endogenous substrates to activated tyrosine kinase through their SH2 domains.

I 438 A FAMILY OF NOVEL PROTEIN THREONINE, TYROSINE PHOSPHATASE GENES, Andrea King, Bradford Ozanne, Alan Ashworth*, CRC Beatson Laboratories, Beatson Institute for Cancer Research, Switchback Road, Bearsden, Glasgow, G61 1BD, UK. *Section of Cell and Molecular Biology, Chester Beatty Laboratories, Institute of Cancer Research, London, SW3 6JB, UK.

Cellular proliferation is potently stimulated by a family of growth factors which specifically interact with receptor protein tyrosine kinases. There are two classes of protein kinases which respond to growth factor stimulation in squamous cell carcinomas. The mitogen-activated protein kinases (MAPKs), which are Ser/Thr kinases enzymatically activated by tyrosine and threonine phosphorylation; and the cyclin-dependent Ser/Thr kinases (cdks) which are cell cycle-regulated requiring tyrosine and threonine dephosphorylation for activation. This opens the possibility for a dual specific protein phosphatase acting as either a tumour suppressor gene, regarding the MAPKs, or indeed as an oncogene in the pathway involving cdks.

Recently a growth factor inducible gene, CL100/3CH134, has been shown to encode a MAP kinase phosphatase *in vitro* [1]. Using this information, highly degenerate oligos were designed to the catalytic and regulatory regions of CL100 and other protein tyrosine phosphatases. PCR was performed on a cDNA library made from the squamous cell carcinoma cell line, A431. The PCR products were then cloned and sequenced. Four clones were found to be homologous, but not identical to CL100, and these were used to screen human liver and brain libraries. This yielded 6 related but clearly distinct cDNAs. The function and pattern of expression of these genes are currently being investigated.

[1]. D.R.Alessi, C.Smythe, S.M.Keyse (1993). Oncogene, 8, 2015-2020.

I 439 HER3 / erbB3 IS PHOSPHORYLATED ON TYROSINE IN BREAST CARCINOMA CELLS IN RESPONSE TO NDF(NEU DIFFERENTIATION FACTOR)

Yoshiko A. Kita, Jenny Barff, Duanzhi Wen, Naili Liu, Sally A. Prigent*, William J. Gullick*, Margery Nicolson, Department of Immunology, Amgen, Amgen center, Thousand Oaks, Ca. 91320 ; * ICRF Oncology Group, Hammersmith Hospital, Du Cane Road, London W12 ONN.

Her3 / erbB3 has been identified as a third member of the epidermal growth factor receptor (EGFR) family. The natural ligand for Her3 has not been identified. Her3 was immunoprecipitated from a lysate of breast carcinoma cell line MDAMB453 using a specific antibody(61.3). By Western blot a ~180 Kd protein was detected when probed with anti-Her3, 49.3. MDAMB453 was stimulated with NDF [isolated by Peles et al (Ref.)] or with NDF isoforms and immunoprecipitated with anti-Her3 (61.3), followed by Western blot and probed with anti-phosphotyrosine. Tyrosine phosphorylation of the ~180kd protein was apparent. Immunoprecipitation of Her2 from the same NDF treated lysate, also revealed tyrosine phosphorylation of pp185 Her2. Whether the tyrosine phosphorylation of Her3 caused by NDF is through direct ligand-receptor interaction or a more indirect mechanism, such as receptor cross-talk, is not clear. In further study of receptor-ligand interaction, receptors were reacted with I^{125} labeled NDF and then chemically crosslinked with BS3. Immunoprecipitation either with anti-Her2 or anti-Her3 revealed dense high MW band (>400Kd). The results suggest that NDF may induce receptor hetero-oligomerization. Further studies utilizing cell lines expressing little or no Her2 will help to clarify the mechanism of the NDF-Her3 interaction.

References

Peles, E., et al (1992) Cell, 69, 205-216
Wen, D., et al (1992) Cell, 69, 1-14
Prigent, S. A., et al (1992) Oncogene, 7,1273-1278

I 440 pp60$^{c\text{-}src}$ TYROSINE KINASE ACTIVITIES; DEPHOSPHORYLATION OF LIGANDS OF THE SH2 DOMAIN. W. B. Knight, D. Kassel, Gampe, R., D.G. Davis, P. Delacy, B. Ellis, T. Gilmer, X. Huang, M. Luther, L. Overton, I. Patel, M. Rodriguez, D. Weigl & D. Willard. Glaxo Res. Inst., RTP, NC, 27709.

We have used a continuous assay for pp60$^{c\text{-}src}$ tyrosine kinase (*src*TK) activity, electrospray ionization mass spectrometry (ESI-MS), and P-31 NMR to examine the autoactivation and the subsequent reaction with peptides and phosphopeptides of a *src*TK construct produced in a baculovirus expression system. The dependence of a MgATP dependent lag in the production of MgADP on *src*TK concentration indicated an intermolecular autoactivation. The ESI-MS spectra before and after MgATP treatment indicated conversion of *src*TK to active *src*TK-P. The inactive *src*TK-P initially present was converted to *src*TK(P)$_2$. The presence of ligands for the SH2 and SH3 domains of *src*TK had little effect on either the rate of autophosphorylation or the final catalytic activity. In fact, TK displayed an apparent phosphatase activity, converting AcY*EEIE, a ligand for the SH2 domain, to AcYEEIE. This was dramatically enhanced upon activation. AcYEEIE was also a substrate for the kinase. The initial rates of both reactions were similar. The apparent phosphatase activity was inhibited by ATP(γ)S and staurosporine, competitive inhibitors versus MgATP of kinase activity (K_i= 20 μM and 10 nM respectively). MgADP did not dramatically stimulate the activity. A construct containing only the SH2 domain displayed weak phosphatase activity which was not activated by MgATP. These data suggest that this activity resides in the noncatalytic domain, but requires an active kinase domain. Additional work is underway to establish the site and mechanism of the apparent phosphatase activity. In addition, activated *src*TK displays slow glycerol kinase and MgATPase activities in the absence of phosphoacceptor peptides. The apparent phosphatase activity suggests new roles for *src*TK in signal transduction.

I 441 SIGNALS INDUCED DURING PRIMING OF HUMAN EOSINOPHILS BY INTERLEUKIN-5. Leo Koenderman, Tjomme van de Bruggen, Deon Kanters, Jan-Willem J. Lammers, and Jan A.M. Raaijmakers. Dept. of Pulmonary Diseases, University Hospital Utrecht, Utrecht, NL-3500 GA, The Netherlands.

Interleukin-5 (IL-5) is a very potent proliferation and differentiation factor for eosinophil precursors. Moreover, this cytokine proofs to be a very potent primer of cell functions of mature eosinophils. These functions include chemotaxis and activation of the respiratory burst (ED_{50} ≈ 10 - 100 pM). The signals, that are essential for IL-5-induced priming of eosinophils, are unknown. However, various signals are induced after addition of IL-5 to these cells. One of the early signals is the phosphorylation of several proteins on tyrosine residues. Most prominent is the phosphorylation of two bands of 102 kDa and 122 kDa as deduced by Western blotting with anti-phosphotyrosine Mab (4G10). It is difficult to relate the phosphorylation events to the priming induced by IL-5, because the commercial available tyrosine kinase inhibitors (e.g. genistein, tyrphostins etc) have different aspecific effects on eosinophils and results obtained by these inhibitors are difficult to interpret. Another intracellular event induced by addition of IL-5 to eosinophils is the induction of a rise in pH_i. This rise is induced by IL-5 in concentrations (10 - 100 pM) that are sufficient for priming. The change in pH_i is induced after a lagtime of ± 10 min and is sustained for a period up to 30 min. The consequences of this cytosolic alkalinization remain to be established. One of the obvious changes induced by IL-5 on the cell membrane is the transient enhancement of avidity of the opsonin receptors FcγRII and CR3. In a classical rosetting assay it was shown that both receptors on eosinophils have an enhanced capacity to bind to IgG and iC3b coated erythrocytes upon addition of IL-5.

I 442 SIGNALING MECHANISMS IN OSTEOCLASTS MEDIATED BY pp60$^{c\text{-}src}$. Joan Levy, Lynn Neff, George Yeh and Roland Baron, Department of Cell Biology, Yale University School of Medicine, New Haven, CT 06510

Deletion of the c-*src* proto-oncogene by homologous recombination in mice is associated with an impairment in osteoclast (OC) function (Soriano, et al 1991). Osteoclasts, in addition to pp60$^{c\text{-}src}$, express high levels of the c-*fyn*, c-*yes* and c-*lyn* gene products. The other non-receptor tyrosine kinases (NRTK's) present in osteoclasts cannot compensate for the loss of *src* function thereby indicating that pp60$^{c\text{-}src}$ has a unique role in this cell type. In order to understand the importance of *src* in regulating osteoclast activity, we tried to identify signaling pathways that *src* may be involved in. Stimulation of authentic mouse or rat osteoclasts with RGD peptides induced a wave of tyrosine phosphorylation at least as determined by quantitative fluorescence confocal microscopy. In order to determine the importance of the *src* gene product in mediating this response, we performed similar experiments in osteoclasts isolated from *src*$^+$ and *src*$^-$ mice. Briefly, isolated osteoclasts were stimulated with RGD-containing peptides for 1-5 minutes, stained with anti-phosphotyrosine antibodies followed by fluorescent secondary antibodies and analyzed by quantitative fluorescence confocal microscopy. Binding of RGD peptide to *src*$^+$ OCs induced a rapid and significant increase in P-tyr reaching maximum levels at 1-3 minutes followed by a return to normal values by 5 minutes. Both mean and maximal P-tyr values increased 1.5-3-fold by 1-3 minutes after addition of RGD peptide ($P<0.01$). In contrast, P-tyr levels were not induced by RGD stimulation of *src*$^-$ OCs. Since *src*$^-$ OCs fail to respond to RGD peptides with a wave of tyrosine phosphorylation, it is concluded that c-*src* expression is required in osteoclasts for integrin-induced tyrosine phosphorylation.

I 443 INTERACTION OF HUMAN pp60$^{c\text{-}src}$ WITH CELLULAR PROTEINS IN HUMAN TUMOR-DERIVED CELL LINES. D. K. Luttrell, A. Lee, T. J. Lansing, R. M. Crosby, K. D. Jung, M. Rodriguez*, J. Berman*, and T. M. Gilmer, Depts. of Cell Biology and Medicinal Chemistry, Glaxo Inc. Research Institute, Research Triangle Park, NC 27709.

Many human malignancies show constitutive activation of tyrosine kinases. Since signal transduction mediated by tyrosine kinases is dependent upon SH2-mediated interactions, inhibition of such interactions may be of therapeutic value in the treatment of cancer. Human breast cancers, for example, often overexpress two closely related receptor tyrosine kinases, the receptor for epidermal growth factor (EGF) and p185$^{HER2/neu}$. Amplification of the genes for these proteins is correlated with poor clinical prognosis. Many primary breast tumors, as well as colon carcinomas, also show elevated activity of the non-receptor tyrosine kinase, pp60$^{c\text{-}src}$, a protooncogene product which has been implicated in the response of cells to EGF.

Since activation of the pp60$^{c\text{-}src}$ kinase has been reported in a number of human tumors, we examined the interactions of the SH2 domain of human c-*src* with target proteins in human tumor-derived cell lines. We cloned the SH2, SH3, and SH3/2 regions of human c-*src* into the glutathione-S-transferase fusion protein vector, pGEX-3X. The GST fusion proteins were used to affinity purify both tyrosine-phosphorylated and non-phosphorylated proteins from human colon and breast carcinoma cell lines. In both normal cells and human breast tumor-derived cell lines, the *src* SH2 domain bound to the focal adhesion kinase, p125^{FAK}, p185$^{HER2/neu}$, and activated EGF receptor. In human colon carcinoma cell lines, the SH2 domain bound to p125^{FAK}, and SHC, an SH2 containing tyrosyl phosphoprotein thought to be involved in the activation of *ras* proteins. Endogenous pp60$^{c\text{-}src}$ tightly associated with tyrosine-phosphorylated EGFR in hormone-stimulated breast carcinoma cell lines, and with SHC in colon carcinoma cell lines. Association of the *src* SH2 with the EGFR was blocked by tyrosyl phosphopeptides containing sequences from the *src* carboxy terminus or the major sites of autophosphorylation in the EGF receptor. GST fusion proteins were engineered with mutations within the SH2 domain, converting arginines 158, 159, and 178 to lysines. These mutations differentially affected the recognition of phosphoproteins in cell lysates. These results raise the possibility that association of pp60$^{c\text{-}src}$ with tyrosyl phosphoproteins may contribute to malignant transformation.

I 444 CLONING OF A TYROSINE PHOSPHATASE EXPRESSED IN RAT SMOOTH MUSCLE CELLS AND CHROMOSOMAL LOCALIZATION OF ITS HUMAN AND MOUSE HOMOLOGUES. David W. Maher, Susanne Edelhoff, Luis G. Borges, Christine Disteche, and Daniel F. Bowen-Pope. Vascular Protein Tyrosine Phosphatase β (VPTPβ) was initially identified as expressed in rat smooth muscle cells by PCR using primers directed towards conserved tyrosine phosphatase regions. A cDNA containing the full coding region was isolated from a rat brain library; sequencing revealed a high level of identity with the recently identified tyrosine phosphatase Syp/PTP1D/SHPTP2/SHPTP3/PTP2C. The VPTPβ protein consists of two src-homology 2 (SH2) domains and a tyrosine phosphatase catalytic domain. A related Drosophila tyrosine phosphatase, *corkscrew*, has been shown to function in conjuction with Drosophila *raf* to transduce a signal from a transmembrane tyrosine kinase during Drosophila embryonic development. VPTPβ has been shown to associate with the cytoplasmic region of a number of activated tyrosine-kinase-containing receptors, including the PDGFβ receptor, HER2-*neu*, *kit*-SCF, and the EGF receptor. VPTPβ is widely expressed, with high levels of the mRNA present in brain, heart and muscle. The chromosomal locations of the human and mouse homologues were determined using a combination of somatic cell hybrid analysis and fluorescent *in situ* hybridization. The human homologue was mapped to region 5p15.1, and the mouse homologue was localized to region 5f. These regions had not previously been shown to be syntenic.

I 445 A NEW MEMBER OF THE FIBROBLAST GROWTH FACTOR RECEPTOR FAMILY CLONED IN BIRDS: ITS EXPRESSION DURING EARLY EMBRYONIC DEVELOPMENT. Christophe Marcelle†, Anne Eichmann, Orna Halevy*, Christiane Bréant and Nicole M. Le Douarin. Institut d'Embryologie Cellulaire et Moléculaire, 94736. Nogent Sur Marne Cedex. France. * The Hebrew University of Jerusalem, Faculty of Agriculture, 76100, Rehovot.Israel.†Present address: Department of Developmental and Cell Biology. University of California, Irvine 92717 CA. USA

We have cloned a new member of the fibroblast growth factor receptor family, FGFR5, from avian embryonic RNA. During elongating primitive streak stages, FGFR-5 is expressed in the rostral and lateral epiblast and in the Hensen's node. From 2.5 days of development (E 2.5) on, it is expressed in various ectoderm- and mesoderm-derived structures. Most striking is FGFR-5 expression in the skeletal muscle lineage. It is highly expressed in the early myotome and, at later stages, in all skeletal muscles of the embryo. From E9 to hatching, FGFR-5 expression in the muscles decreases dramatically but is maintained in satellite cells of adult muscles. We conclude that FGFR5 may play multiple roles in early avian development, including a specialized role in the early differentiation of skeletal muscle.

I 446 INTERACTIONS BETWEEN TYPE I AND TYPE II ACTIVIN RECEPTORS, Lawrence S. Mathews, Department of Biological Chemistry, University of Michigan, Ann Arbor, MI 48109

The protein growth and differentiation factor, activin, binds to cell surface receptors of approximately 55 and 75 kDa, which are known as the type I and type II receptors, respectively. Two closely related type II receptors, ActRII and ActRIIB, have been shown to be transmembrane protein serine kinases, and belong to a subfamily of kinases that includes receptors for the transforming growth factors β and the bone morphogenetic proteins. We have recently demonstrated that a new member of this family can act as an activin type I receptor (ActRI; K. Tsuchida et al., PNAS in press). Affinity labeling with radiolabeled activin of cells transfected with both receptors revealed that ActRI and ActRII form a stable complex. The dependence of dimer formation on ligand binding, as well as on different domains of the receptors, is currently under investigation. Futhermore, coexpression of ActRI and ActRIIB allows for activin-dependent phosphorylation of specific sites on ActRIIB. Those phosphorylation sites are being mapped, and their functional importance in activin signaling will be determined.

I 447 PROTEIN HISTIDINE PHOSPHATASE ACTIVITY IN RAT LIVER AND SPINACH LEAF EXTRACTS, Harry R. Matthews, Department of Biological Chemistry, University of California, Davis, CA 95616

Histidine phosphorylation is a major post-synthetic modification of proteins in cells. For example, in *Physarum* nuclei about 5% of the ^{32}P incorporated into proteins in vivo in a 2 h. label (25% of the generation time) is found in phosphohistidine. In several eukaryotic systems, protein histidine kinase has been demonstrated and a protein histidine kinase has been purified from budding yeast. The catalytic subunits of several known protein phosphatases (including PP1, PP2A, PP2C, PPX) are excellent protein histidine phosphatases. The K_{cat}/K_M values for phospho-histidine are equal to or greater than those for phospho-serine. Protein phosphatases 1 and 2A are found in the cell as heteromeric holoenzymes. The holoenzyme forms of PP1 and PP2A were separated by ion-exchange chromatography. In a rat liver cytosolic extract, one peak containing PP1 and two peaks containing PP2A were found, as previously described. In a spinach leaf extract, three peaks containing PP1 and one peak containing PP2A were resolved. All of these peaks were active protein histidine phosphatases. In both extracts, there was evidence for additional phosphatase activity specific for phosphohistidine, including a peak containing PPX in the rat liver extract.

Most of this work was carried out in Professor Philip Cohen's laboratory, The University, Dundee, Scotland. It was support-ed by the American Cancer Society and a Yamagiwa-Yoshida Fellowship from the International Union Against Cancer.

I 448 REGULATION OF C-JUN N TERMINAL PHOSPHORYLATION BY EXTRACELLULAR SIGNALS, Minden, A. Lin, A., Smeal, T., Hibi, M., and Karin, M. University of California at San Diego. La Jolla, CA 92093

c-Jun, encoded by the c-jun proto-oncogene, is an important component of the transcriptional activator AP-1 and appears to have a central role in regulation of cell proliferation, differentiation, and neoplastic transformation. The activity of c-Jun is negatively regulated by phosphorylation at three sites near the C terminal DNA binding domain, while phosphorylation at two sites at the N terminal activation domain (serines 63 and 73) positively regulates its activity. A serine/threonine kinase, JNK, has been identified, which binds cJun and phosphorylates serines 63 and 73 in response to a variety of different extracellular stimuli. JNK is regulated differently from the extracellular signal regulated kinases (ERKs) in response to different stimuli. JNK can be activated by stimuli which do not activate ERKs, while ERKs can be activated by agents which do not activate JNK. Both enzymes, however, are activated by oncogenic Ha-Ras and depend normal Ha-Ras function for proper activation by external stimuli. This work provides evidence for two parallel signaling cascades that transmit information from the cell surface through Ha-Ras to the transcriptional machinery in the nucleus. Signal transduction in response to cell stimulation may therefore be more complex than previously expected. Rather than triggering a linear cascade of events, cell surface stimulation probably triggers multiple converging and diverging kinase cascades which ultimately transmit information to the nucleus.

I 449 USE OF THE TWO-HYBRID SYSTEM TO ISOLATE COMPONENTS OF THE INTERLEUKIN-1 SIGNALLING PATHWAYS, Jennifer L. Mitcham, Margit A. Gayle and John E. Sims, Immunex Corporation, 51 University Street, Seattle, WA 98101

We are applying the two-hybrid protein interaction cloning system (Fields & Song, 1989) to isolate proteins that interact with the cytoplasmic domain of the interleukin 1 receptor. Though the receptor and ligand members of the IL-1 system have been characterized, little is known about mechanisms of IL-1 signalling or components of IL-1 signal transduction pathways. Isolation of proteins that physically interact with the cytoplasmic domain of the IL-1 receptor would allow identification and characterization of components of the IL-1 signalling pathway. We constructed a vector encoding a fusion protein consisting of the DNA-binding domain of the yeast transcriptional activator, GAL4, and the cytoplasmic domain of the human IL-1 receptor (IL-1R) and introduced this vector into the yeast reporter strain, Y190. Expression of this fusion protein in Y190 has been confirmed by western blot. We have also constructed a library encoding GAL4 activation domain/human cDNA fusion proteins using RNA from the IL-1 responsive KB human epidermoid carcinoma cell line. We are presently screening this library by transformation into Y190 carrying the IL-1R fusion vector. Theoretically, positive clones will arise if the activation domain/human protein fusion encoded by a particular library vector interacts with the DNA-binding domain/IL-1R fusion. The interaction of the two fusion proteins reconstructs the GAL4 transcriptional activator, driving expression of a histidine selectable marker and a β-gal reporter gene.

Fields, S., and Song, O. *Nature* 340, 245-246 (1989).

I 450 CLONING AND CHARACTERIZATION OF A TYPE I RECEPTOR FOR TGF-β, Kohei Miyazono, Petra Franzén, Hidenori Ichijo, Hidetoshi Yamashita, Peter Schulz, Peter ten Dijke, and Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Box 595 Biomedical Center, S-751 24 Uppsala, Sweden

Transforming growth factor (TGF)-βs exert their effects through binding to two different types of signaling receptors, i.e. type I (TβR-I; 53 kd) and type II (TβR-II; 75 kd). TβR-I and TβR-II are indispensable for TGF-β signaling; TβR-II is needed for the binding of TGF-β to TβR-I, and TβR-I is required for the signal transduction induced by TβR-II. Similar to the activin type II receptors, TβR-II has a serine/threonine kinase domain in its cytoplasmic portion. Thus, serine/threonine kinase receptors form a new receptor family, which include the receptors for the proteins in the TGF-β superfamily.

By a PCR-based approach, we have obtained six novel serine/threonine kinase receptors, termed activin receptor-like kinase (ALK)-1 through 6. They have overall structures similar to that of TβR-II. One of the clone, ALK-5, was shown to bind ^{125}I-TGF-β1 after transfection into porcine endothelial cells, and formed a cross-linked complex of 70 kd, which is characteristic of TβR-I. Immunoprecipitation of the cross-linked complexes by antibodies against ALK-5 revealed the 70 kd complex as well as the co-immunoprecipitated 94 kd TβR-II complex.

Other ALKs could also bind ^{125}I-TGF-β1 after co-transfection with the TβR-II cDNA into COS cells, and therefore, they may also serve as type I receptors for TGF-βs. However, the TβR-I complex in several cell types, including mink lung epithelial cells, could be most efficiently precipitated by the ALK-5 antibodies, suggesting that ALK-5 appears to be the most important TGF-β type I receptor in these cells. Transfection of the ALK-5 cDNA into TβR-I-deficient cells restored TGF-β-induced PAI-1 production. These results suggest that signal transduction by TGF-β involves the formation of a heteromeric complex of two different serine/threonine kinase receptors, i.e. TβR-I/ALK-5 and TβR-II.

I 451 PROTEIN TYROSINE KINASE DEPENDENT AND INDEPENDENT PATHWAYS IN TNF-alpha MEDIATED CYTOTOXICITY AND DNA FRAGMENTATION, Hideki Morimoto and Benjamin Bonavida, Department of Microbiology and Immunology, University of California at Los angeles, UCLA School of Medicine, Los Angeles, CA 90024

We have recently reported that TNF-alpha (TNF-a) synergizes in cytotoxicity against sensitive and resistant human tumor cell lines. We examined whether the pathway of TNF-mediated cytotoxicity is the same when TNF-a is used alone or when used with toxins. The role of protein tyrosine kinases (PTK) in cytotoxicity was examined using the MTT assay in a sensitive human ovarian tumor cell line 222 and its TNF-resistant variant 222TR. Both 222 and 222TR were sensitive to the cytotoxic effect of combination treatment with TNF-a and diphtheria toxin (DTX), and synergy was obtained. Genistein, a PTK inhibitor had no effect on DTX-mediated cytotoxicity. Genistein inhibited the sensitivity of 222 to TNF-a, and the synergistic cytotoxicity with TNF-a and DTX on 222 was markly reduced but was not abolished. In contrast, there was no inhibitory effect by genistein on the low cytotoxicity by TNF-a or the synergy achieved by TNF-a and DTX in the 222TR line. Since both TNF-a and DTX trigger apoptosis as measured by the DNA fragmentation assay, we examined the effect of genistein on the induction of apoptosis. Genistein had no effect on DTX-mediated apoptosis. However, there was significant inhibition of DNA fragmentation induced by TNF-a in the 222 sensitive line. Also, there was significant inhibition of DNA fragmentation in the synergy with TNF-a and DTX in the 222 line but no detectable inhibitory effect on synergy in the 222TR line. Altogether, these findings demonstrate that TNF-mediated cytotoxicity can proceed by two distinct pathways, namely a PTK-dependent and a PTK-independent pathway. In the sensitive line, both pathways are operational whereas in the TNF-resistant 222TR line only the PTK-independent pathway is active. Further, the findings on the DNA fragmentation corroborated those obtained in the cytotoxicity system. These results imply that one mechanism of tumor cell resistance to TNF-a involves the PTK-signaling pathway.

I 452 DOWN REGULATION OF THE EGF RECEPTOR BY MAP KINASE. Peter Morrison and Marsha Rich Rosner. Unversity of Chicago, Chicago, IL 60637. Down regulation of the epidermal growth factor (EGF) receptor following EGF stimulation is an important mechanism for controlling cell growth. One possible means by which the receptor may be targeted for degradation is direct phosphorylation of the EGF receptor at specific sites. The threonine 669 (Thr669) residue is the major phosphorylation site in the EGF receptor, it is a substrate for MAP kinase, and its phosphorylation is increased by EGF and MAP kinase activators. To test the hypothesis that phosphorylation of Thr669 is involved in EGF receptor down regulation, we constructed a mutant EGF receptor with a phosphothreonine mimic at residue 669 (glutamic acid, Glu669) and determined the effect of this mutation on EGF receptor turnover. Analysis of stable transfectants in Chinese Hamster Ovary (CHO) cells showed a dramatic decrease in the levels of Glu669 receptor relative to the wild type EGF receptor following EGF stimulation. In contrast, substitution of glutamic acid at threonine 654, the protein kinase C phosphorylation site, yielded a receptor with stability comparable to the wild type EGF receptor. Degradation of surface-labeled Glu669 EGF receptor was also enhanced relative to the wild type EGF receptor, whereas the rate of synthesis of the mutant receptor upon long-term exposure to EGF was decreased relative to the wild type. These results suggest a new mechanism for down regulation of EGF-stimulated EGF receptor involving phosphorylation at threonine 669 by MAP kinase.

I 453 ALTERED SIGNAL TRANSDUCTION BY A MUTANT EGF RECEPTOR FROM HUMAN TUMORS, David K. Moscatello, Laural B. Ludlow, Marina Holgado-Madruga, and Albert J. Wong, Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, PA 19107.

Significant advances have recently been made in understanding the components and interactions of the signal transduction cascade of the EGF receptor (EGFR). We have studied a naturally occurring mutant form of the EGFR that is found in 17% of glial tumors and 25% of non-small cell carcinoma of the lung tumors. Overexpression of this protein in NIH-3T3 cells results in ligand-independent transformation. We now report that the pattern of tyrosine phosphoproteins is altered in these cells compared with that seen in EGF-stimulated cells overexpressing the normal EGFR and that the levels of proteins associated with EGFR signal transduction are altered in clones overexpressing this mutant EGFR. Western blot analysis indicates that the level of rasGAP in two independent clones transformed by this mutant EGFR is substantially elevated in comparison with clones overexpressing the normal EGFR or transfected with vector only. A 100 kDa form of rasGAP is found in several clones expressing the mutant EGFR that is undetectable in clones expressing normal EGFR or transfection controls. Conversely, the level of GRB2, recently shown to link the EGFR with mSos, is decreased in these clones. The level of SHPTP2/syp is substantially increased in these cells with a cross-reactive 120 kD form also being found. In contrast, no differences in the levels of the 85 kD subunit of PI-3 kinase, Nck, or PLC_γ were detected in any of the clones examined. While PLC_γ associates with the normal EGFR in EGF-stimulated cells, no association of the mutant EGFR could be demonstrated either by immunoprecipitation or western blotting with the SH2 domain of PLC-γ. These results indicate that overexpression of the mutant EGFR can alter the expression of other signal transduction proteins, and these changes may provide insight into the components of signal transduction relevant to cellular transformation.

I 454 cDNA CLONING OF AN ETS FAMILY PROTEIN, PEA3β, WHOSE TRANSACTIVATION ACTIVITY IS ENHANCED BY RAS OR RAF-1, Koichi Nakajima, Kazuto Nakae and Toshio Hirano, Division of Molecular Oncology, Biomedical Research Center, Osaka University Medical School, Osaka 565 Japan

The transcription factors of Ets proto-oncogene family recognizing purine-rich target sequences have been implicated in transformation and other cellular functions through transcriptional activation of a number of genes often in response to extracellular signals or oncogenes including *fms*, *src*, *ras* and *raf-1*. We have isolated a cDNA clone that encodes an ubiquitously expressed ETS-family member by RT-PCR using a set of degenerative primers corresponding to the conserved region in the ETS DNA binding domain, followed by screening a HepG2- and a human placenta-cDNA libraries. The cDNA clone named PEA3β encodes a polypeptides of 520 amino acids with an Ets DNA binding domain highly similar to those of PEA3 and ER81. We found that the transcriptional activity of PEA3β was enhanced by activated Ha-Ras, or Raf-1, and the induced-activity of PEA3β was greatly diminished by a dominant-negative form of Raf-1, RafC4. Transactivation domain of PEA3β was localized within the N-terminal 166 amino acids using a GAL4-fusion protein system. The transactivation domain consists of an acidic region and a proline-rich region. Three putative phosphorylation sites for MAP kinases in the proline-rich region may be responsible for Ras or Raf-induced activation. Thus the ubiquitously expressed PEA3β may be a nuclear target factor mediating extracellular signals in a variety of cells including embryonic cells.

I 455 A RECEPTOR PROTEIN TYROSINE PHOSPHATASE INVOLVED IN GROWTH CONE REGULATION IN THE MEDICINAL LEECH

Michael N. Nitabach and Eduardo R. Macagno, Department of Biological Sciences, Columbia University, New York, New York, 10027.

In order to correctly navigate through a developing embryo, neuronal growth cones must respond appropriately to extracellular cues by turning, stopping, resuming growth, etc. This implies the existence of mechanisms located in the growth cone which transduce extracellular interactions into intracellular signals which can trigger these responses. One class of molecule that may be involved in this process are the receptor protein tyrosine phosphatases (rPTPases), which are transmembrane proteins containing extracellular motifs like those of adhesion molecules and cytoplasmic PTPase catalytic domains. Interaction of the extracellular region with its ligand(s) could regulate the enzymatic activity of the PTPase domains and thereby affect growth cone behavior. Recent studies have shown the accumulation of rPTPase mRNAs in developing neurons of both vertebrates and invertebrates, and the localization of rPTPase proteins to embryonic *Drosophila* axons, but have not directly implicated rPTPases in growth cone function. To begin to address this issue, we cloned two leukocyte antigen-related (LAR) rPTPases from the medicinal leech, *Hirudo medicinalis*: *Hm*LAR1 and *Hm*LAR2. *In situ* hybridization experiments show that each of these is expressed in a distinct segmentally iterated subset of central neurons. In addition, *Hm*LAR2 mRNA accumulates in a segmentally iterated bilateral pair of giant cells which elaborate a parallel array of 70 processes, each of which terminates in a very large growth cone. Immunocytochemistry with an *Hm*LAR2-specific antiserum shows that *Hm*LAR2 protein accumulates to high levels in these giant growth cones. Its temporal pattern of accumulation suggests that *Hm*LAR2 may be involved in triggering and/or maintaining rapid elongation by these growth cones.

I 456 SHC BINDS TO TYR1148 OF ACTIVATED HUMAN EGF RECEPTOR AND MEDIATES RAS SIGNALING, Yoshinori Okabayashi, Yoshiaki Kido, Toshio Okutani, Yutaka Sugimoto, Kazuhiko Sakaguchi and Masato Kasuga, Second Department of Internal Medicine, Kobe University School of Medicine, Kobe 650, Japan

Autophosphorylation of receptor tyrosine kinase provides binding sites for signaling proteins containing SH2 domain. Shc is SH2-containing adaptor protein that associates with EGF receptors. In the present study, we investigated the specific interaction between EGF receptors and Shc and the role of Shc in Ras signaling, using CHO cells overexpressing mutated EGF receptors in which autophosphorylation sites were replaced by phenylalanine. Cells were stimulated with 30 nM EGF for 2 min and lysed. Cell lysates were immunoprecipitated with anti-EGF receptor antibody and immunoblotted with anti-Shc antibody. Shc proteins of 66, 52 and 46 kd were co-immunoprecipitated with wild-type EGF receptors. Binding of Shc to the EGF receptor mutants lacking Tyr1148 was markedly decreased, whereas the mutants retaining Tyr1148 bound the wild-type level of Shc. The same results were obtained when the association of Shc to the immunoprecipitated and in vitro phosphorylated EGF receptors was examined. Then, we examined the ability of the synthetic peptides representing Tyr1148 to block the association of GST-Shc SH2 domain fusion protein to the in vitro phosphorylated wild-type EGF receptors. The phosphorylated nonameric peptide, DNPNpYQQDF, inhibited the binding of GST-Shc to the receptor, whereas phosphorylated pentameric peptide, pYQQDF, inhibited the binding, suggesting that N-terminal sequences adjacent to phosphotyrosine are necessary for the association of Shc. To investigate the role of Tyr1148 on mitogenic signal transduction, we measured the EGF-induced activation of Ras protein in CHO cells overexpressing EGF receptor mutants in which only Tyr1148 was retained or all five Tyr residues were replaced. Although both mutants activated Ras protein, activation of Ras was greater in the mutant in which Tyr1148 was rescued. These results indicate that Tyr1148 of the activated human EGF receptor is a major binding site of Shc and the binding of Shc to Tyr1148 is implicated in Ras signaling.

I 457 GENETIC CHANGES OF THE TYPE II TGF-ß RECEPTOR GENE IN GASTRIC CANCER CELLS : CORRELATION WITH THE SENSITIVITY TO THE GROWTH INHIBITION BY TGF-ß Keunchil Park, Anita B. Roberts, Yung-Jue Bang*, Jae-Gahb Park*, Noe Kyeong Kim* , Michael B. Sporn and Seong-Jin Kim, Laboratory of Chemoprevention, National Cancer Institute, Bethesda, MD 20892, * Seoul National University Medical College, Seoul, Korea

Transforming growth factor-ßs (TGF-ßs) are prototypic multifunctional negative growth factors that inhibit the growth of virtually every lineage of cells and also regulate cell differentiation and the expression of extracellular matrix proteins. However, very little is known about the mechanisms whereby the TGF-ßs mediate these many effects. Cross-linking to radiolabeled to TGF-ß has revealed three types of TGF-ß receptors at the cell surface, i.e., Type I, II, and III TGF-ß receptors. With the recent molecular cloning of these receptors, it is now known that type I and II receptors are transmembrane receptors containing cytoplasmic serine/threonine kinase domains, while type III receptor has a short cytoplasmic tail with no obvious signaling motif. In the present study we show that there are several genetic changes of the type II TGF-ß receptor gene in gastric cancer cells which are resistant to the growth inhibitory effect of TGF-ß. Southern blot analysis demonstrated deletion of the type II receptor gene in 2 out of 7 gastric cancer cells, and amplification in another 2 gastric cancer cells. On the other hand, one gastric cancer cell line which is sensitive to growth inhibition by TGF-ß did not show any abnormalities of the type II receptor gene. In addition, Northern blot analysis revealed that some of the gastric cancer cells that are resistant to the growth inhibitory effect of TGF-ß express either a truncated type II TGF-ß receptor mRNA or no detectable mRNAs , whereas the cell line that retains responsiveness to TGF-ß expresses a full-size type II receptor mRNA. Our results suggest that one of the possible mechanisms of escape from negative autocrine or paracrine growth control by TGF-ß could involve various genetic changes of the type II receptor gene itself or altered expression of its message possibly by transcriptional abnormalities.

I 458 CONTROL OF CELL FATE BY THE *DROSOPHILA* PROTEIN TYROSINE PHOSPHATASE, *CORKSCREW*, Lizabeth A. Perkins*, Michael Melnick+ and Norbert Perrimon+, *Pediatric Surgical Research Laboratories, Massachusetts General Hospital, Boston, MA 02114, +Dept. of Genetics, Harvard Medical School, Boston, MA 02115

Genetic, developmental and molecular studies have demonstrated that *corkscrew* (*csw*) functions maternally in the receptor tyrosine kinase (RTK) mediated "terminal class" pathway which is required for the establishment of cell fate determination of embryonic terminal structures. Several terminal class genes have been characterized and they include the RTK *torso*, a GTP binding protein encoded by *ras-1*, a guanine nucleotide exchange factor encoded by *SOS*, the kinases *D-raf* and *Dsor1*, and the transcription factors *tailless* and *huckebein*. The PTPase *csw* is a positive transducer of the terminal class signal and acts downstream of the RTK *torso* and upstream of the transcription factors *tailless* and *huckebein*. In addition to *csw's* putative catalytic activity, it also encodes two SH2 domains, as well as an SH3 binding site, suggesting that *csw* can mediate heteromeric protein interactions.

Additionally, *csw* is required zygotically. Normally, homozygous males derived from heterozygous females die during pupal stages; however, homozygous male and female adult flies can be obtained under appropriate genetic conditions. These homozygous *csw* adult flies exhibit several mutant phenotypes: antennae and legs are missing their most distal structures, the aristae and claws, respectively; the eyes exhibit reduced numbers of ommatidia and wing veins L4 and L5 do not extend to the distal wing margin. Further, the chorions of eggs derived from homozygous *csw* females display a ventalized phenotype. The wing and chorion phenotypes might be attributed to *csw* functioning in the DER pathway (*Drosophila* homologue of the EGFR). However, cell fate determination of distal segments of appendages has not, as yet, been correlated with a RTK mediated pathway.

Finally, *csw* is functionally conserved through evolution. Two mammalian proteins, *PTP-1C* (*SH-PTP1*, *HCP*, *SHP*) and *SH-PTP2* (*Syp*, *PTP-1D*) which are structurally similar to *csw*, have been tested *in vivo* for their functional homology to *csw*. *SH-PTP2* was found to rescue *csw's* pupal lethality while the maternal lethality was only partially rescued. No functional overlap was observed with *PTP-1C*.

Analysis of *csw's* gene structure, as well as RNA analyses have suggested that alternatively spliced *csw* proteins, which exhibit both temporal and tissue specific regulation, interact with differing constellations of proteins throughout development.

I 459 **ANALYSIS OF RET ONCOGENES SIGNAL TRANSDUCTION PATHWAY**, M.A. Pierotti, P.G. Pelicci*, G. Pelicci*, I. Bongarzone, E. Arighi, L. De Filippis and M.G. Borrello - Istituto Nazionale Tumori, Milano and *Istituto Clinica Medica I, Policlinico Monteluce, Universita' di Perugia, Perugia.

RET protooncogene encodes a tyrosine kinase receptor-like transmembrane protein. Oncogenic rearrangements of RET tk domain with different 5' "activating" genes have been frequently detected in human papillary thyroid carcinomas. Moreover, germ-line mutations in the genomic portion coding for the extracellular domain of RET protooncogene have been recently associated with the inherited cancer syndrome MEN2A.

The proto-RET ligand has not yet been identified, therefore the signal transduction triggered by RET tyrosine kinase has not been explored. However, since we have determined that following gene rearrangements the oncogenic versions of RET product are phosphorylated on tyrosine and display a constitutive tyrosine kinase enzymatic activity, we began the analysis of RET signal transduction by investigating the biochemical properties of its oncoproteins. We have found that different RET oncoproteins bind in vitro the SH2 domains of the adaptor proteins Grb2 and Shc. Moreover, in living cells, Shc proteins are phosphorylated and coimmunoprecipitate with RET oncoproteins and with Grb2 protein. PLC-gamma was also found in anti-Shc and in anti-RET immunoprecipitates. The analysis of other elements of the signal transduction pathway is now in progress.

I 460 A NOVEL MEMBRANE ASSSOCIATED FORM OF PROTEIN PHOSPHATASE 2A DEPHOSPHORYLATES THE β-ADRENERGIC RECEPTOR. Julie Pitcher and Robert J. Lefkowitz. HHMI. Department of Medicine, Duke University Medical Center, Durham, NC 27710.

The reversible phosphorylation of G protein coupled receptors is one mechanism whereby receptor function is regulated. Although considerable progress has been made in identifying and investigating the receptor kinases involved in this desensitization process, the nature of the phosphatases responsible for reversing these events remains obscure. We have characterized β-adrenergic receptor phosphatase activity (βARPh) in extracts of bovine brain utilizing as a substrate purified reconstituted β2AR phosphorylated with the agonist specific kinase β-adrenergic receptor kinase (βARK). Surprisingly, βARPh activity is only observed when assays are performed in the presence of protamine or after freeze/thawing the crude extract in the presence of β-mercaptoethanol, two conditions known to activate protein phosphatase 2A (PP-2A). No βARPH activity is observed when either Mg2+ or Ca2+/calmodulin are added to crude extracts. The activity observed after freeze/thawing is not inhibited by I-2 (an inhibitor of protein phosphatase 1) but is potently inhibited by okadaic acid (IC50=1nM). These observations are consistent with the identification of the βARPh activity as an oligomeric, low activity form of PP-2A. Interestingly, when utilizing purified forms of PP-2A neither the catalytic subunit or PP-2A0 (AB'C) are capable of dephosphorylating βARK phosphorylated βAR, while both PP-2A1 (ABC) and PP-2A2 (AC) have significant receptor phosphatase activity. Unlike other reported forms of PP-2A, βARPh activity is tightly associated with the membrane. Solubilization of the membranes with detergent reveals βARPh activity in the absence of protamine. This activity, which gel-filters with a molecular weight of approximately 35kDa., presumably represents the free catalytic subunit of PP-2A. Removal of detergent converts this soluble receptor phosphatase to a latent form which exhibits activity only in the presence of protamine and gel-filters with an apparent molecular weight of 120 kDa. In the presence of protamine the receptor phosphatase:phosphorylase a phosphatase activity ratio of the latent phosphatase is approximately 5-fold greater than that of the free catalytic subunit. These data suggest that βARPh is a novel membrane associated oligomeric form of PP-2A. The factors responsible for activating this latent phosphatase are currently under investigation.

I 461 IDENTIFICATION OF SEQUENCES DETERMINING MEMBRANE OR NUCLEAR LOCALIZATION IN MEMBRANE-ASSOCIATED AND NUCLEAR VARIANTS OF THE C-FGR TYROSINE KINASE, Jeffrey W. Potter, Walter A. Duran, Tao-Lin Yi, and Cheryl L. Willman, Departments of Pathology and Cell Biology, University of New Mexico School of Medicine, Albuquerque, NM 87131

Our previous studies have determined that C-FGR, a member of the SRC family of non-receptor tyrosine kinases, is selectively expressed in myeloid cells (PNAS 86:4254; Oncogene 4:1081; Blood 77:726). We have also determined that the murine C-FGR gene encodes two proteins through alternative translation initiation (J. Cell. Biochem. 16B:219). A full-length 59 kd c-fgr protein (pp59c-fgr) containing the amino terminal, SH2, SH3, and kinase domains arises from translation initiation at the first in-frame AUG. In contrast, a truncated 53kd c-fgr protein (pp53c-fgr) arises from internal translation initiation from an AUG in the 3' end of the amino terminus and contains only the SH2, SH3, and kinase domains. Both c-fgr proteins are detectable in COS cells transiently transfected with a c-fgr cDNA, stably transfected fibroblasts, normal monocytic cells, and monocytic cell lines. While pp59c-fgr associates with the actin-containing cytoskeleton in the plasma membrane, pp53c-fgr is located in the nucleus (where it associates with the chromosome kinetichores at mitosis) and perinuclear membranes. Mutation/deletion of the G^2K^7 amino terminal myristylation sequence in pp59c-fgr results in translocation of pp59c-fgr to the nucleus, in a pattern identical to pp53c-fgr. Thus, the c-fgr amino terminal myristylation sequences function in part to inhibit nuclear localization of wild type pp59c-fgr. Scanning of the c-fgr protein sequence revealed two domains with homology to known nuclear localization sequences (NLS). The first candidate domain (^{187}KIRKLD^{192}) homolgous to the NLS of SV40 large T antigen, is localized in the c-fgr SH2 domain and is conserved in 6/9 members of the SRC family. A second candidate domain (K^{407}FPIK^{412}) homolgous to the NLS of yeast mating factor α2, is located within the c-fgr kinase domain and is highly conserved in all members of the SRC family. When either candidate NLS was individually mutagenized or deleted in pp53c-fgr or in the myristylation mutant of pp59c-fgr and mutant constructs were transiently transfected into COS cells, nuclear localization was only partially diminished; mutagenesis of the kinase domain NLS (KFPIK) had a more significant effect than the SH2 domain NLS (KIRKLD). When both sequences were mutagenized or deleted in pp53c-fgr or in the myristylation mutant of pp59c-fgr, nuclear localization was completely abolished and both proteins displayed a cytoplasmic/perinuclear staining pattern. These data imply that variant c-fgr tyrosine kinases are likely to play important functional roles in both the nucleus and the cytoplasm.

I 462 INACTIVATION OF PROTEIN KINASES ACTING ON TRANSLATION FACTORS BY INSULIN AND GROWTH FACTORS. Christopher G. Proud, Nicholas T. Redpath, Gavin Welsh and Andrea Flynn, Dept. of Biochemistry, University of Bristol, Bristol BS8 1TD. UK.

Insulin and growth factors rapidly activate mRNA translation in animal cells. Our work concerns the roles of changes in the phosphorylation and activities of translation initiation and elongation factors (eIF's and eEF's, respectively) in these effects.

Insulin and growth factors lead to inactivation of two protein kinases which act on translation factors. The first of these is glycogen synthase kinase-3 (GSK-3) which phosphorylates the largest subunit of (ε) the guanine nucleotide exchange factor eIF-2B, leading to inactivation of the factor and to impairment of peptide chain initiation. eIF-2B is acutely activated by insulin and by other stimuli, and phosphorylation of eIF-2B by GSK-3 appears to play an important role in these effects. GSK-3 itself is apparently inactivated by phosphorylation, and we will present evidence concerning the signal transduction pathways involved.

Elongation factor eEF-2 is specifically phosphorylated by a Ca/CaM-dependent protein kinase termed eEF-2 kinase. Phosphorylation completely inactivates eEF-2. Insulin and growth factors cause rapid dephosphorylation of eEF-2 and hence increased rates of peptide-chain elongation, and this is associated with inactivation of eEF-2 kinase. We will report progress in elucidating the mechanisms through which eEF-2 kinase is regulated by insulin, and in cloning this kinase.

The inactivation of GSK-3 and of eEF-2 kinase provide mechanism through which insulin can activate, respectively, peptide-chain initiation and elongation.

I 463 p34*cdc2* CATALYZES THE PHOSPHORYLATION AND INACTIVATION OF MAP KINASE KINASE 1, Anthony J. Rossomando[1], Paul Dent[2], Thomas W. Sturgill[2] and Daniel R. Marshak[1], [1]W.M. Keck Structural Biology Laboratory, Beckman Neuroscience Center, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724 [2]Department of Pharmacology, University of Virginia, Charlottesville, VA 22908

MAP kinase kinase 1 (MKK1), a dual specificity tyrosine and threonine protein kinase, has been shown to be phosphorylated and activated by the raf oncogene product as part of the mitogen-activated protein kinase cascade. Here we report the phosphorylation and inactivation of MKK1 by p34*cdc2*, a serine/threonine protein kinase that regulates the cell division cycle. MKK1 contains a consensus phosphorylation site for p34*cdc2* at Thr-286 and a related site at Thr-292. p34*cdc2* catalyzes the phosphorylation of MKK1 on both of these threonine residues and inactivates the MKK1 enzymatic activity *in vitro*. Both sites are also phosphorylated *in vivo* during mitosis, and co-immunoprecipitation studies suggest the p34*cdc2* kinase is responsible for the phosphorylation *in vivo*. This inhibition indicates an interaction between the intracellular program for cell cycle control and extracellular mitogenic signals.

I 464 RECEPTOR TYROSINE PHOSPHATASE RPTP-κ CAN MEDIATE HOMOPHILIC CELL BINDING

Jan Sap, Ying-Ping Jiang, and Joseph Schlessinger, Department of Pharmacology, NYU Medical Center, 550 First Avenue, New York, NY 10016.

Receptor protein tyrosine phosphatase (RPTPase) RPTP-κ is a widely expressed cell surface protein displaying several features of classical cell adhesion molecules. Its extracellular moiety is proteolytically processed, and contains a combination of domain signatures, including a single MAM domain, as well as an Ig domain and four fibronectin type III domains. The latter are related to those found in the *tie* receptor tyrosine kinase. Its intracellular juxtamembrane domain bears similarity to the intracellular domains of cadherins, and is followed by two tandemly repeated phosphatase homology domains.

The similarities between RPTP-κ and cell adhesion molecules prompted us to study the capacity of this RPTPase to mediate homophilic cell binding. We demonstrate that expression of RPTP-κ mediates the ability of transfected indicator cells to aggregate in a homophilic manner. Aggregation does not require phosphatase activity, or proteolytic processing of the extracellular domain. In addition RPTP-κ expressing cells show adhesive binding to substrates coated with recombinant purified extracellular domain of RPTP-κ. Moreover, binding is specific, in that no binding is observed between RPTP-κ and the related RPTPase mRPTPμ (collaboration with M. Gebbink, G. Zondag, and Dr. W. Moolenaar, Amsterdam). These observations suggest that RPTPases provide a link between specific intercellular contact/adhesion and cellular signalling pathways involving tyrosine phosphorylation.

I 465 **INSULIN REGULATION OF HEPATOCYTE SH2-DOMAIN PTP**, Susan M. Sell and David E. Reese, Clinical Diabetes and Nutrition Section, NIH, NIDDK, Phoenix, AZ 85016

FAO cells are an insulin-sensitive, rat-derived hepatoma cell line. Both insulin receptor isotypes are expressed in these cells. FAO cells provide a model system for analyzing immediate early responses to insulin, since we have shown that insulin receptor isotype distribution changes in these cells within 15 min of insulin treatment (1).

It has also been reported that this cell line exhibits a drop in soluble protein tyrosine phosphatase (PTP) activity following insulin stimulation (2). Multiple PTP are likely to be involved in the insulin signalling cascade. SH2-domain PTP are a class of PTP selected as candidates for regulating tyrosine phosphorylation following insulin stimulation since PTP are thought to be involved in receptor-mediated tyrosine kinase signalling pathways (3). Using degenerate oligonucleotides, the SH2-domain hematopoietic PTP, PTP1C (4), was found to be expressed in these cells. Sequence analysis confirmed the identification of this PTP as PTP1C.

The expression of the SH2-domain PTP exhibited a decrease in expression following insulin treatment. We will present our findings on PTP1C protein expression and phosphatase activity in FAO cells following insulin stimulation.

References
1)Sell, S.M., et al. 1993, submitted
2)Goldstein, B.J., et al. 1991 Adv. Prot. Phosphatases 6:1-17.
3)Pawson, T. et al. 1992 Cell 71:359-362.
4)Shen, S.H., et al. 1991 Nature 352:736-739.

I 466 SECRETORY PROCESSING OF THE AMYLOID PRECURSOR PROTEIN OF ALZHEIMER'S DISEASE IS STIMULATED BY ACTIVATION OF PROTEIN KINASE C AND TYROSINE KINASES. B.E. Slack, J. Breu, K. Srivastava, and R.J. Wurtman, Department of Brain and Cognitive Sciences, MIT, Cambridge MA 02139

The amyloid precursor protein (APP) of Alzheimer's disease (AD) is a large, membrane-spanning molecule whose extracellular domain (APP^s) is released following cleavage at a site external to the membrane. Disruptions in the normal processing of APP may contribute to the formation of amyloid deposits found in the brains of AD patients. It was previously shown that release of APP^s is stimulated by the muscarinic agonist carbachol in human embryonic kidney (HEK) cells transfected with human muscarinic receptor subtypes m1 or m3, but not in cells expressing m2 or m4 receptors (Nitsch et al., Science 258:304,1992). The m1 and m3 subtypes are coupled to phosphatidylinositol hydrolysis, while the m2 and m4 subtypes are not. The response to carbachol was inhibited by the protein kinase inhibitor staurosporine, suggesting the involvement of protein kinase C (PKC) in the processing pathway. However, recent evidence now suggests the involvement of tyrosine kinases as well. Carbachol stimulated tyrosine phosphorylation of two proteins of approximately 66 and 106 kDa, respectively, in HEK cells. The effect was more pronounced in cells expressing m3 than m2 muscarinic receptor subtypes. Peroxides of vanadate, which inhibit protein tyrosine phosphatases, stimulated release of APP^s as effectively as did carbachol, and direct activation of PKC with phorbol esters. While stimulation of APP^s release by all three treatments was blocked by the tyrosine kinase inhibitor genistein, the specific PKC antagonist GF109203X blocked only the response to phorbol esters. The results suggest that both PKC and protein tyrosine kinases participate in the regulation of APP processing. Abnormalities in the activities of PKC and tyrosine kinases in AD have been reported, and may contribute to amyloidogenesis in this disease. (Grant no. MH-28783 of NIMH).

I 467 BINDING SITES IN THE EGF RECEPTOR FOR SH2-CONTAINING PROTEINS, Concepció Soler, Laura Beguinot and Graham Carpenter, Department of Biochemistry, Vanderbilt University, School of Medicine, Nashville, TN 37232.

Tyrosine autophosphorylation of the epidermal growth factor (EGF) receptor creates binding sites for important signal tranduction molecules that contain *src* homology 2 domains (SH2), such as phospholipase C-γ1 (PLC-γ1), *ras* GTPase-activating protein (*ras*GAP), p85 subunit of phosphatylinositide 3-kinase (PI-3K) and *src* homology and collagen (SHC). To examine which autophosphorylation sites of the EGF receptor were involved in the *in vivo* interaction with PLC-γ1, *ras*GAP, p85 and SHC, cells expressing EGF receptor mutants with autophosphorylation sites mutated or removed by truncation were used. The extent of association of either PLC-γ1, *ras*GAP, p85 or SHC with the EGF receptor was not modified by single mutation of any autophosphorylation site (tyrosines 1173, 1148, 1086, 1068, 992). Significant reduction of the EGF receptor association with these substrate was obtained when tyrosines 1173, 1148 and 1068, major autophosphorylation sites of EGF receptor, were simultaneous mutated or deleted by truncation of carboxy-terminal receptor residues. EGF receptor did not associate with these substrates when all five autophosphorylation sites were mutated or removed by truncation. Taken together, these results indicate that there is not a unique single autophosphorylation site essential for the *in vivo* association of PLC-γ1, *ras*GAP, p85 or SHC with the EGF receptor, rather non-specific or compensatory sites seem to be involved.

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I 468 ELEVATION OF INSULIN RECEPTOR PHOSPHOPEPTIDE PTPase ACTIVITY IN LIVER OF INSULIN-RESISTANT ob/ob MICE, Janet Sredy, Diane R. Sawicki, Donald Sullivan, and Brenda R. Flam, Wyeth-Ayerst Research, Princeton, NJ 08543

The ob/ob mouse, an animal model of non-insulin dependent diabetes mellitus (NIDDM), is hyperinsulinemic, hyperglycemic, obese and insulin-resistant. The inability of ob/ob tissues such as liver to respond to insulin is associated with a reduction in insulin induced protein tyrosine phosphorylation. To ascertain whether this decrease in phosphorylation was due to increased phosphatase activity, PTPase activity was determined in particulate and soluble fractions from livers of ob/ob and age-matched control mice. The synthetic phosphopeptide TRDIY(P)ETDY(P)Y(P)RK, corresponding to the major sites of autophosphorylation of the insulin receptor was used as the substrate. Mice of 5 to 27 weeks of age were evaluated. In both ob/ob and control livers most of the PTPase activity was found in the particulate fraction. The particulate PTPase activity was significantly higher in the ob/ob, irrespective of age. The soluble PTPase activity in the ob/ob was significantly below control activity at 5-7 weeks but increased with age and reached values significantly above control at 21-23 weeks. The plasma insulin was greatly elevated in the ob/ob across all age groups while the plasma glucose was highest in the 5-7 week ob/ob and declined with age. Thus, despite euglycemia in the 21-23 week ob/ob, these mice were hyperinsulinemic and had elevated hepatic PTPase activities. In vitro, the PTPase activity was sensitive to inhibition by vanadate, zinc, and molybdate and was not appreciably affected by high concentrations of fluoride, okadaic acid, tetramisole, or tartrate. In conclusion, the ob/ob mouse has elevated PTPase activity that may cause a decrease in insulin mediated protein tyrosine phosphorylation and contribute to the decline in insulin action in NIDDM.

I 469 INSULIN AND PHORBOL ESTER STIMULATE MAP KINASE KINASE KINASE ACTIVITY OF RAF-1

Kojiroh Ueki*¶, Satoshi Matsuda†, Kazuyuki Tobe*, Yukiko Gotoh†, Hiroyuki Tamemoto*, Makoto Yachi*, Yasuo Akanuma¶, YoshioYazaki*, Eisuke Nishida† & Takashi Kadowaki*

* Third Department of Institute of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

† Department of Biophysics and Biochemistry, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, Japan

¶Institute of Diabetes Care and Research, Asahi Life Foundation, Marunouchi, Chiyoda-ku, Tokyo 100, Japan

Raf-1 kinase (Raf-1) has recently been implicated in $p21^{ras}$-mediated signaling pathway and has been reported to have mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK) activity in the cells either expressing v-raf or overexpressing c-raf. Here we show that the MAPKKK activity of the anti- Raf-1 antibody (α-raf) immunoprecipitates maximally increases 2 min after insulin stimulation in Chinese hamster ovary cells overexpressing human insulin receptor (CHO-HIR cells), while the maximal activities of MAP kinase kinase (MAPKK) and MAP kinase (MAPK) are reached approximately 5 min after the stimulation, suggesting the presence of kinase cascade of Raf-1 , MAPKK and MAPK in insulin-stimulated signaling pathway *in vivo* . We also show that MAPKKK of Raf-1 is activated by the protein kinase C activator 12-*O*-*tetradecanoyl*-phorbol-13-acetate (TPA) in the time course similar to that by insulin. These results suggest that signals initiated by insulin and TPA stimulation converge on Raf-1 and activate its MAPKKK activity, presumably via activation of $p21^{ras}$ and PKC, respectively. Moreover, insulin and TPA also cause the hyperphosphorylation of Raf-1 and MAPKK (MKK1, not MKK2), this hyperphosphorylation proceeds more slowly (maximal phosphorylation at ≅10 min) than their kinase activities. Furthermore, overexpression of MAPK results in enhanced hyperphosphorylation of Raf-1 and MKK1. These results suggest that Raf-1 and MAPKK not only lie upstream of MAPK but also are phosphorylated by feedback mechanism.

I 470 IDENTIFICATION OF HUMAN ACTIVIN AND TGF-β TYPE I RECEPTORS THAT FORM HETEROMERIC KINASE COMPLEXES WITH TYPE II RECEPTORS, Francesc Ventura, Liliana Attisano, Juan Cárcamo, Frances M.B. Weis, Joan Massagué and Jeffrey Wrana. Cell Biology and Genetics Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

The members of the TGF-β superfamily of growth and differentiation factors interact to a set of cell surface proteins. Among them, both receptor types I and II have been shown to be required for signalling. Type II receptors, which contain a cytoplasmic serine/threonine kinase domain, bind ligand on its own. Also, type II receptors are required for ligand binding to the type I receptors, with which forms a heteromeric receptor complex.

We have found that two human transmembrane serine/threonine kinases, Act R-I and TSR-I, distantly related to TGF-β and activin type II receptors, have the properties of type I receptors for these factors. Both Act R-I and TSR-I bind activin in concert with activin type II receptors, with which both are able to form heteromeric complexes. However, only ActR-I mediates a particular transcriptional response upon activin activation, suggesting functional differences between related type I receptors. TSR-I can also bind TGF-β in concert with the TGF-β type II receptor, indicating that type I receptors can be shared by different members of the TGF-β superfamily. These results show that type I receptors are transmembrane protein kinases that associate with the type II receptors to generate diverse heteromeric serine/threonine kinase complexes of different signalling capacity.

I 471 PHOSPHORYLATION AND ACTIVATION OF TYPE-1 PROTEIN PHOSPHATASE BY cdc2 AND F_A/GSK3, Emma Villa-Moruzzi and Franca Puntoni, Department of Biomedicine, University of Pisa, 56126, Pisa, Italy

Type-1 protein phosphatase (PP1) is regulated by hormones and growth factors and during cell cycle. An inactive form of PP1, a complex of catalytic subunit and inhibitor-2 (E_i•I2) is activated through phosphorylation of I2 by the kinase F_A/GSK3. Recently we found that also cdc2, the kinase that is active at mitosis (p34•cyclin B complex, purified from mitotic HeLa cells), activates inactive PP1. Likewise the case of F_A/GSK3, PP1 activation by cdc2 is accompanied by phosphorylation of I2 and free I2 can be phosphorylated as well. Both activation and phosphorylation by cdc2 are specific (and not due to contaminating F_A/GSK3) since they are inhibited by a cdc2-substrate peptide which does not affect F_A/GSK3. Mono-dimensional phospho-peptide maps of phosphorylated I2 using three different proteases showed that different peptides were obtained when I2 was phosphorylated by F_A/GSK3 or cdc2. Also bi-dimensional phospho-peptide maps showed that after tryptic proteolysis two peptides (a and b) were obtained with I2 phosphorylated by F_A/GSK3, while two new peptides in addition to a and b were obtained from cdc2-phosphorylated I2. However, when the inactive PP1 (E_i•I2) was phosphorylated only peptide a and b were obtained from I2, with differences between the two kinase: mainly peptide a, which contains only Thr-P, was obtained with F_A/GSK3, while peptide b, which contains both Thr-P and P-Ser, was prevalently obtained from I2 phosphorylated with cdc2. This difference might explain both the higher I2 phosphorylation obtained with cdc2 than with F_A/GSK3 and the fact that dephosphorylation by PP1 (either endogenous or added exogenously) was very rapid in the case of F_A/GSK3-phosphorylated I2, while some of the phosphate introduced by cdc2 was much more resistant to dephosphorylation by either PP1 or PP2. One additional difference between the two kinases is that cdc2 (and not F_A/GSK3) phosphorylates also the catalytic subunit of PP1, whether isolated or in the complex with I2. The phosphorylation is on a Thr located in the trypsin-sensitive, C-terminal region. Since cdc2 is activated at mitosis we labelled "in vivo" mitotic HeLa cells with [32]Pi. We found that in mitotic (but not in asynchronous) cells the immunoprecipitated PP1 was phosphorylated on the catalytic subunit.

I 472 ANALYSIS OF PTP 1D INTERACTION WITH THE $p185^{HER2/neu}$ ONCOGENE PRODUCT AND ITS SIGNIFICANCE FOR BREAST CANCER CELL GROWTH. Wolfgang Vogel and Axel Ullrich, Department of Molecular Biology, Max-Planck-Institut of Biochemistry, 82152 Martinsried, Germany.

The phosphotyrosine specific phosphatase PTP 1D (syp, SH-PTP2) contains two SH2 domains and was shown to bind to activated receptor tyrosine kinases (RTKs) including the PDGF receptor (PDGF-R), EGF receptor and the oncogene product $p185^{HER2/neu}$. Interaction with the PDGF-R kinase results in phosphorylation on tyrosines and activation of the PTP 1D phosphatase function.

To investigate the role of PTP 1D in RTK-mediated signal transduction we focused on its interaction with $p185^{HER2/neu}$ and its potential participation in deregulated breast cancer cell growth. Interestingly, the analysis of mRNA expression in mammary tumor cell lines by Northern blotting revealed a striking correlation of PTP 1D and $p185^{HER2/neu}$ overexpression. This was confirmed on the protein level and the two gene products were found to coimmunoprecipitate from cell lysates. Moreover, using GST-fusion proteins we identified the N-terminal SH2 domain as the primary interaction site with the HER2/neu gene product and an enhancement of binding affinity by the second SH2 domain suggesting an cooperative conformational effect.

The significance of $p185^{HER2/neu}$/PTP 1D interaction for breast cancer cell growth and the identity of PTP 1D substrats are currently under investigation.

I 473 THE YEAST PHEROMONE RESPONSE PATHWAY: ACTIVATION OF STE12

Katja Wassmann, Hans Winkler and Gustav Ammerer
Institute f. Biochemistry and Mol. Cell Biology,
Dr. Bohr-Gasse 7, 1030 Vienna, Austria

The STE12 protein is a transcriptional activator of conjugation-specific genes in *Saccharomyces cerevisiae*. Transcriptional activation of these genes is induced by exposure to mating pheromone (i.e., α-factor) and a DNA sequence called the pheromone response element, which is located in the upstream regulatory region of inducible genes.

It has been demonstrated that STE12 is a late- acting component in the pheromone response pathway. Several kinases are involved in this pathway and they are thought to function before STE12 in the order: STE11, STE7, FUS3/KSS1. It seems likely that the activity of STE12 is controlled by phosphorylation catalysed by one or more of these kinases, most probably FUS3.

Our group is interested in the mechanism of activation of STE12. We could show that the concentration of STE12 and its localisation in the nucleus does not change upon α-factor treatment of the cells. Kinase assays demonstrate that STE12 is a substrate for FUS3 *in vitro*. We also present *in vivo* footprinting analysis and *in vivo* ^{32}P labelling data of the STE12 protein.

I 474 CLONING OF A PUTATIVE HUMAN PHOSPHATIDYLINOSITOL 3- KINASE HOMOLOGUE, Karen Wong, Lewis C. Cantley, Harvard Medical School and Division of Signal Transduction, Beth Israel Hospital, Boston, MA 02115.

An important role for phosphatidylinositol 3-kinase (PI 3- kinase) in transformation is suggested by prior work showing the elevation in levels of PI-3,4-P2 and PI-3,4,5-P3 in virally transformed cells. The finding that PI 3-kinase also associates with activated growth factor receptor tyrosine kinases further implicates its role in mitogenic signaling.

The purified PI 3-kinase consists of 85 kDa and 110 kDa subunits. Subsequent cloning of a bovine p110 cDNA demonstrated that p110 is the catalytic subunit. Bovine p110 shares significant homology with Vps 34, a yeast PI 3-kinase required for proper targeting of vacuolar proteins. Bovine p110 also has substantial similarities to Tor 2, another yeast gene that confers rapamycin resistance of certain strains.

Here we present a full length human cDNA, isolated from a placenta library, that shares homology to these three genes. It has all the conserved residues at the catalytic domain that are known to be required for PI 3-kinase activity. Northern analysis demonstrated its ubiquitous expression in all human tissues tested. We are expressing the full length cDNA in baculovirus and also in COS cells to test its phosphatidylinositol kinase activity.

I 475 Isolation And Characterization of *eps* 8: A Novel Substrate For EGF Receptor And Other Tyrosine Kinases, William T. Wong, Paola Castagnino, Francesca Fazioli and Pier Paolo Di Fiore, Laboratory of Cellular and Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

We have raised antisera specific for phosphotyrosine-containing proteins whose phosphorylation is triggered by the EGF receptor. These antisera were used to screen a NIH 3T3 cell cDNA library and identified a cDNA clone which encoded a 97 KDa protein, termed *eps* 8. We showed that *eps* 8 was phosphorylated by the activated EGF receptor tyrosine kinase as well as other tyrosine kinases. *eps* 8 binds to the EGF receptor directly despite the absence of a SH2 domain. We have further determined the regions of *eps* 8 which are important for its interaction with the EGF receptor. Overexpression of recombinant *eps* 8 in fibroblasts or hematopoietic cells increases these cells' response and sensitivity to the mitogenic action of EGF. Therefore, *eps* 8 is likely involved in the transduction of EGF signals. Sequence analysis revealed a SH3 domain in *eps* 8. We have identified a 65 KDa protein that binds to the SH3 domain of *eps* 8 and showed that this protein is mainly distributed in the nuclear fraction of the cell.

Poster Session V

I 500 A novel IL-6 inducible DNA binding factor which translocates into nucleus by tyrosine phosphorylation. Y. Nishio, M. Inoue, *M. Naruto, #T. Kishimoto, and S. Akira. Institute for Molecular and Cellular Biology, Osaka University; *Basic Research Laboratories, Toray Industries; #Osaka University Medical School

During inflammation, interleukin-6(IL-6) released by different cell types, including monocytes, fibroblasts, and endothelial cells, stimulates the synthesis and secretion of a set of plasma proteins(acute phase proteins) by the liver. Production of acute phase proteins is regulated mainly at a transcriptional level in the liver. Two types of IL-6 response elements have been identified. One represents a binding site for members of the C/EBP family. Of this family, NF-IL6(also called C/EBPβ, AGP/EBP, LAP, IL-6DBP) and NF-IL6β(also called C/EBPδ) were shown to be implicated in the regulation of acute phase protein genes by IL-6. The other consists of a hexanucleotide motif, CTGGGA. However, little is known about transcription factors binding to this motif. In this study we have characterized a nuclear factor specifically binding to the hexanucleotide motif. This factor was detected in a gel retardation assay within 15 minutes after stimulation of IL-6. Furthermore, activation of this factor occurs in the cytoplasm, followed by translocation to nucleus. The most interesting aspect may be that the factor is directly phosphorylated by a tyrosine protein kinase as in the case of ISGF3 activation by interferon. Purification and cloning of the CTGGGA binding factor is now in progress.

I 501 THE OSTEOGENIC GROWTH PEPTIDE (OGP) IS ESSENTIAL FOR OSTEOBLASTIC AND FIBROBLASTIC CELL GROWTH. Itai Bab, Zvi Greenberg, Michael Chorev, Arie Shteyer, Malka Namdar, Andras Muhlrad, Bone Laboratory and Departments of Oral Biology and Oral and Maxillofacial Surgery, The Faculty of Dental Medicine and Department of Pharmaceutical Chemistry, The Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem, Israel, 91010

We have recently discovered a 14 amino-acid osteogenic growth peptide (OGP), identical to the C-terminus of histone H4 [Bab et al (1992) EMBO J., 11:1867]. The OGP was purified from the osteogenic phase of post-ablation marrow regeneration. When administered *in vivo* in rats, the OGP increases bone formation and trabecular bone density in a manner resembling the systemic increase in osteogenesis that accompanies the post-ablation bone marrow healing. Immunoreactive OGP (irOGP) is present physiologically in the serum, mainly in the form of an OGP-OGP binding protein (OGPBP) complex. A marked increase in serum bound and unbound OGP accompanies the marrow post-ablation osteogenic phase and associated systemic osteogenic response. *In vitro* OGP is a potent mitogenic promoter of osteoblastic and fibroblastic cells. In osteoblastic MC 3T3 E1 and ROS 17/2.8 cells and in NIH 3T3 fibroblasts, OGP stimulates proliferation dose dependently at 10^{-14}-10^{-13} M, 10^{-10}-10^{-8} M and 10^{-11}-10^{-10} M, respectively, followed by an inhibition at higher concentrations. **These narrow OGP dose ranges and the peptide's high abundance in serum suggested that the effect in culture of exogenously added OGP is superimposed on a background of an endogenous OGP-like activity.** Here we describe the accumulation of high OGP levels in serum free culture medium of these cells and the isolation of OGP from human serum and medium conditioned with the osteoblastic MC 3T3 E1 cells in which the OGP functions in an autocrine/paracrine circuit. Withdrawal of the endogenous OGP from the medium of these cell systems using anti-OGP antibodies resulted in growth arrest which can be restored to a limited extent by bFGF, IGF-I or PDFG. The endogenous OGP functions synergistically with the bFGF or IGF-I and additively with the PDGF. **These findings indicate that OGP is a ubiquitous stromal cell factor essential for regulating baseline proliferation.**

I 502 EFFECT OF VARIOUS KINASE/PHOSPHATASE BLOCKERS ON GROWTH CONE COLLAPSE EVOKED BY CNS MYELIN-ASSOCIATED NEURITE GROWTH INHIBITORS. Christine E. Bandtlow and Martin E. Schwab. Brain Research Institute, Univ. Zurich, August-Forel-Str. 1, CH-8029 Zurich, Switzerland.

The myelin-associated protein NI-35 from rat central nervous system (CNS) is known to inhibit regeneration of lesioned neuronal fiber tracts in vivo and growth of neurites in vitro. We have recently demonstrated that growth cones of cultured rat dorsal root ganglion neurons arrested their growth and collapsed when exposed to liposomes containing NI-35. Direct measurements of free intracellular calcium concentrations, $[Ca^{2+}]i$, revealed a rapid and large rise in $[Ca^{2+}]i$ within growth cones when exposed to NI-35-containing liposomes. Dantrolene, a blocker of caffeine-sensitive intracellular calcium stores protected growth cones from NI-35-evoked collapse. In addition, depletion of these caffeine-sensitive stores prevented the NI-35-induced increase of $[Ca^{2+}]i$. These results had suggested that NI-35 exerts its neurite growth inhibitory effect through a series of undefined processes that lead to a release of Ca^{2+} from intracellular stores.

Experiments are presently in progress to study the specific effect of various tyrosine- and threonin/serine-kinase and -phosphates blockers on the protection of NI-35-evoked growth cone collapse. Preliminary data indicate that the NI-35-evoked growth cone collapse is sensitive to threonin/serine-phosphatase inhibitors but not to tyrosine-phosphatase inhibitors. Further characterization of the signalling pathway induced by NI-35 will help to understand how NI-35 elicits its biological function as a neurite growth inhibitor.

I 503 IDENTIFICATION OF A NATURALLY OCCURRING MUTANT OF THE dsRNA-DEPENDENT PROTEIN KINASE (PKR) TUMOUR SUPPRESSOR IN A LYMPHOCYTIC LEUKEMIA John C. Bell and Ninan Abraham Department of Medicine, University of Ottawa, 451 Smyth Road, Ottawa, Canada, K1H 8M5

Evidence suggests that the dsRNA-dependent protein kinase (Tik, p68 or PKR) may function as a tumour suppressor. While numerous viral mechanisms for inactivation of PKR have been elucidated, there is no evidence for inactivation of PKR in tumours.

Our findings indicate that Tik, a member of the family of dual specificity kinases that we had cloned based on its anti-phosphotyrosine reactivity, is the murne dsRNA-dependent kinase. Reactivity with antiphosphotyrosine antibodies is conserved between mouse and human PKR suggestive of a dual specificity function for PKR. We have found that the PKR gene has undergone a rearrangement in the lymphocytic leukemia cell line L1210. In this cell line one allele of PKR encodes a truncated PKR polypeptide which acts as a dominant negative mutant and constitutes the first demonstration of PKR inactivation in tumours. Unlike cells with normal levels of PKR, L1210 cells have increased growth in soft agar following interferon treatment. These observations have important clinical implications for the use of interferon as an anti-tumour agent.

I 504 ALTERATION IN THE SIGNAL TRANSDUCTION MACHINERY IN ANTI-IG RESISTANT VARIANTS OF WEHI-231 B LYMPHOMA CELL.

L. E. Benhamou, U. Hibner*, P. A. Cazenave and P. Sarthou.
Institut Pasteur, Unité d'Immunochimie Analytique, Paris, France;* Institut de Génétique Moléculaire de Montpellier, UMR 9932 F34033, Montpellier, France.

Cross-linking of membrane-bound Immunoglobulins (mIgs) by antigen or anti-Ig antibodies in B lymphocytes induces a cascade of intracellular events which controls cell growth and differentiation.

We have used the murine WEHI-231 B lymphoma as a model of tolerance induction in immature B cells. In this cell line mIg ligation leads to Programmed Cell Death (PCD) or apoptosis (1). In order to study the intracellular pathway of PCD induction, we have derived two variants of WEHI-231which are resistant to anti-Ig treatment. Their mIg binding characteristics (number of sites, Kd) are identical to those of the wild-type WEHI-231. After mIg cross-linking the variants do not undergo apoptosis although this process is inducible by a Ca^{2+}-ionophore.

We have analyzed early Ca^{2+} signals. MIg ligation induces an initial Ca^{2+} peak of reduced intensity in the variants than in the wild-type. The following sustained phase is similar in both variants and wild-type.

We have also compared the patterns of intracellular tyrosine-phosphorylated protein generated by mIg cross-linking. These patterns appear to be qualitatively and quantitatively different. The major difference is the markedly reduced expression of a 75kDa protein in the variants. We have identified this protein as HS1. HS1 has been shown to play an important role in B lymphocyte signal transduction.

We have studied c-myc expression patterns, later in the signaling cascade. They differe in the wild-type and the variants both in unstimulated and in anti-Ig stimulated state (2).

Altogether, these results show that alterations in the transduction signal machinery might be responsible for differences in the cellular response: cell death or cell survival.

(1) L. E. Benhamou, P. A. Cazenave and P. Sarthou Eur. J. Immunol., 1990, 20:1405-1407.
(2) U. Hibner, L. E. Benhamou, M. Haury, P. A. Cazenave and P. Sarthou, Eur. J. Immunol., in press.

I 505 *BCL*-2 IS IMPORTANT IN CONTROLLING CELL DEATH DURING THE DEVELOPMENT OF THE NERVOUS SYSTEM. Ora Bernard and Peter Farlie, The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia

Apoptosis, also known as programmed cell death, is an active process which is thought to be involved in controling cell numbers in various tissues of different species during embryonal development and throughout adult life. It is estimated that half of all neurons produced during embryogenesis die before adulthood. Although the mechanisms governing apoptosis are as yet unknown, it is suggested that in neurons cell death takes place when the number of neurons reaching their targets exceed the optimal number. In this case the concentration of target-derived neurotrophic factors available to the neuronal population is not sufficient to support their survival and therefore results in their death. The *bcl*-2 proto-oncogene product protects different cell types from apoptotic cell death and is expressed in the developing and adult nervous system. To study the role of *bcl*-2 in regulation of neuronal cell death we generated transgenic mice expressing *bcl*-2 in neurons under the control of the neuron specific enolase promoter. These transgenic mice express high levels of the *bcl*-2 transgene uniquely in neurons. Cultured sensory neurons isolated from dorsal root ganglia of newborn mice depend for their survival on nerve growth factor, however when these cells were isolated from the *bcl*-2 transgenic mice they survived in culture in the absence of neurotrophic factors. The number of different types of neurons in the central and peripheral nervous system was found to be at least 50% higher in the *bcl*-2 transgenic mice than in normal mice indicating the importance of *bcl*-2 in controlling neuronal survival during development.

I 506 **MOLECULAR CLONING AND EXPRESSION OF A *SACCHAROMYCES CEREVISIAE* GENE ENCODING A PROTEIN WITH ATPASE ACTIVITY AND POTENTIAL HELICASE ACTIVITY THAT HAS HOMOLOGY WITH THE HUMAN IMMUNODEFICIENCY VIRUS *TAT* BINDING PROTEIN-1.**

†Chojnicki, E. W. T. and ‡Lucero, H., †Bristol-Myers Squibb Co., Pharmaceutical Research Institute, Analytical Research and Development, P.O. Box 4755, Syracuse NY 13221-4755. ‡Boston University School of Medicine, Department of Physiology, 80 East Concord Street, Boston, MA 02118-2394.

Genes which encode proteins having a high degree of homology with Tat-binding protein-1 have recently been identified in widely divergent evolutionary species. The proteins encoded by these genes are localized in the nucleus and cytoplasm. Those localized in the nucleus have highly conserved domains associated with nucleotide binding and DNA/RNA helicases, suggesting that these proteins exhibit these activities in their native state. The cloning of a new member of this family of genes, YT3P, from *Saccharomyces cerevisiae* is described. The purified recombinant protein product was obtained by expression in E. coli. It exhibited a molecular weight of 46 kD as determined by SDS/PAGE in agreement with that predicted from its DNA sequence. It is a Mg^{++} dependent ATPase that is stimulated five fold by single stranded DNA. However, YT3P protein poorly catalyzed DNA unwinding when assayed using a 28-base nucleotide primed to M13 single stranded DNA as partial duplex substrate. This is the first direct evidence that the putative homologies to ATPases and helicases observed in this family of proteins may indicate actual physiologic functionality.

I 507 **THE PDGF INDUCIBLE FACTOR SIF IS RELATED TO THE INTERFERON ACTIVATED P91 TRANSCRIPTION FACTOR.** Brent H. Cochran[1], Leisa K. Johnson[1], Neptune Mizrahi[1], Deepa Bhavsar[1], Xin-Yuan Fu[2], Andrew C. Larner[3], David S. Finbloom[3].

[1]Department of Physiology, Tufts University School of Medicine, Boston, MA 02111, [2]Dept. of Biochemistry, Mount Sinai School of Medicine, New York, NY 10029, [3]Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD 20892.

We have previously identified a PDGF-inducible DNA-binding protein, which binds to a conserved sequence approximately 346 bp upstream of the c-*fos* gene. We have called this factor SIF, for *sis*/PDGF-inducible factor. This DNA-binding activity is rapidly activated upon treatment of quiescent cells with *sis*/PDGF and occurs in the absence of new protein synthesis. Upon induction by PDGF, the intensity of SIF DNA binding increases and new bands appear on band shift gels. UV crosslinking and purification of the protein indicates that an approximately 91 kD protein is the DNA-binding component of SIF. We have investigated whether SIF might be related to the interferon stimulated ISGF3 transcription factor that has some similar properties. We have found that an antibody to the C-terminus of the 91 kD subunit of ISGF-3 will supershift SIF DNA-binding activity. SIF binds to sequences closely related to that of the γ interferon activated elements, but not to the α, β interferon activation sequence the ISRE. Interferon γ induces SIF DNA binding activity and this induction is abolished in cell lines defective for gene encoding ISGF3-p91. Thus, we conclude that the 91 kD subunit of the interferon stimulated transcription factor ISGF3 or a closely related factor is an integral component of the SIF DNA binding complex.

I 508 IDENTIFICATION OF GENOMIC SEQUENCES REQUIRED FOR THE INDUCTION OF STROMELYSIN BY NERVE GROWTH FACTOR IN PC12 CELLS, Sunita deSouza, Cindy M. Machida, Janis E. Lochner and Gary Ciment, Department of Cell Biology and Anatomy, Oregon Health Sciences University, Portland, OR 97201

Stromelysin-1 (ST-1) is an extracellular matrix metalloprotease whose expression is induced by nerve growth factor (NGF) in rat PC12 cells. In this study, we examine NGF-responsive DNA regulatory sequences in the 5'-untranslated region of the ST-1 gene in PC12 cells. We found that NGF induces ST-1 mRNA expression at least 1000-fold over initially undetectable levels. We found, moreover, that this induction was due to *de novo* transcription and was sensitive to cycloheximide, indicating that ST-1 is a "late" gene. Transient transfection assays using a plasmid containing a 750 base pair region of the 5'-untranslated region of the ST-1 gene fused to the CAT reporter gene indicated that this region contained NGF-responsive cis-acting elements. We have performed transient transfection assays using various manipulations of the ST-1 promoter region. We found that mutations of an AP-1 sequence within this promoter was necessary for both basal and NGF-induced levels of CAT gene expression. 5'-deletion mutational analysis revealed that sequences between -247 and -315 bp upstream of the transcriptional start site are necessary for NGF-responsiveness. Using mobility-shift assays, we have recently shown that sequences present in this region bind to nuclear protein(s) and that this DNA-protein interaction is both specific and saturable. These studies suggest that the induction of the ST-1 gene in PC12 cells may be a useful system for studying the NGF-signalling pathway.

I 509 **NITRIC OXIDE AND GENE EXPRESSION IN NEURONS,** Grigori Enikolopov and Natalia Peunova, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

Signals that ultimately produce changes in synaptic plasticity can also elicit modifications of gene activity patterns in neurons. Induced changes in gene activity may in turn be required for long-term changes in neuronal function. We are interested in whether the gaseous messengers nitric oxide (NO) and carbon monoxide (CO) can stimulate gene activity. We have tested a series of reporter genes and found that NO can act as an amplifier of calcium signals in neurons provided that these two types of signals coincide in time. The effect of NO on signaling might be particularly important at very low levels of calcium action, at which the inducer acting alone would have a negligible effect; these very weak signals might be amplified by NO, resulting in pronounced physiological changes for the cell. The results of experiments with reporter promoters, chimeric activator proteins and recombinant protein kinase inhibitors implicate the PKA-CREB-CRE system as a major component of the signaling pathway for the transcriptional synergy of NO and Ca^{2+}. We have applied a similar strategy to study the effect of CO in neurons and to investigate the cooperation or antagonism of these two signaling systems. We shall also present data on the connection between NO production and the differentiation of neuronal cells.

I 510 REGULATION OF SEPTUM FORMATION IN *S.POMBE* : THE ROLE OF THE *cdc7* PROTEIN KINASE
Christian Fankhauser, and Viesturs Simanis, Cell Cycle Unit, Swiss Institute for Experimental Cancer Research, CH-1066 Epalinges
There are a number of *S. pombe* mutants which exhibit phenotypes consistent with a defect in septum formation and cytokinesis. Mutations in the early septation genes *cdc7, 11, 14* and *15* prevent formation of the division septum. However, growth, DNA synthesis and mitosis continue in the absence of cytokinesis, so the cells become highly elongated and multinucleate. A defect in the *cdc16* gene leads to the formation of multiple septa indicating that the controls limiting the cell to the production of one septum per cell cycle are operative. *Cdc16*$^+$ is also required for maintenance of high levels of the *cdc2* kinase activity during mitosis. The gene has been cloned and encodes a homologue of the *S. cerevisiae* mitotic checkpoint *BUB2*. The *cdc14* gene is required for cytokinesis and acts as a mitotic inhibitor when overexpressed. These results suggest that the early septation genes and *cdc16* are not only required for cytokinesis but also for its coordination with other cell cycle events, in particular mitosis. We have recently cloned the *cdc7*$^+$ gene and found that it is an essential protein kinase. Its role during cytokinesis and interactions with other key elements of mitosis and cytokinesis will be presented.

I 511 **ARREST OF NERVOUS CELL LINES PROLIFERATION AT THE G2/M TRANSITION OF THE CELL CYCLE BY PROTEIN PHOSPHOTYROSINE PHOSPHATASE (PTP) INHIBITION.**
Robert Faure, Michel Dufour, Alan Shaver**, Barry, I. Posner*. Ontogénèse et génétique moléculaire centre hospitalier de L'université Laval Québec. Department of *medicine and **chemistry, Mc Gill university, montreal.
Peroxydes of vanadium (pVs) are PTP inhibitors characterized on endosomal insulin and epidermal growth factor receptors associated PTP(s) (Faure et al.,1992 J. Biol. Chem. 267: 11215). In order to evaluate the final consequence of a general PTP(s) inhibition on cultured cells, we have studied the effects of two stable peroxovanadium derivatives recently synthesized: bis pV[1, 10 phenanthroline] (bpV[phen]) and bis pV[2-carboxypyridine] (bpV[pic]) on the proliferation of two nervous cell lines: a neuronal cell line (NB 41) and a glial cell line (C6). In the presence of bpV(phen) the proliferation of NB41 cells was markedly reduced to 30 % of the control value after 72 hrs of culture (dose, 2 µM). We have estimated the percentage of cells in each phase of the mitotic cycle by measuring the intracellular DNA content using laser flow cytometry. We observed that cells accumulated at the G2/M transition as a function of time (24-72 hrs) and dose (0.25-2 µM): after 72 hrs of culture, 2 µM bpV (phen): 67 ± 5.9 % in G2/M, N = 5; controls: 21. 1 ± 5.6 %, N = 5; viability > 95 %. At a higher dose (5 µM) similar results were obtained with the C6 glial cell line. The same effects but for 10 times larger doses were observed with bpV(pic) a compound which had the same inhibitory potency in an in vitro PTP assay. The G2/M block was reversible as cells returned to the G1 phase after removing the inhibitor. No DNA damages were observed in a sensitive nicks detecting assay. $pp34^{cdc2}$ kinase expression measured by western blotting using a specific antibody was not affected in treated cells however tyrosine hyperphosphorylation of $pp34^{cdc2}$ was detected with a concomitant reduction (70 %) in kinase activity towards histone H1. Cells morphology was not altered as judged by phase contrast microscopy and indirect immunofluoresence using a vimentin antibody. **In conclusion** i) A consequence of a long term and general inhibition of PTP activity is a greatly reduced cell proliferation due to the inhibition of PTP(s) involved in the control of the mitotic cycle ii) sensitivity to pV derivatives differs according to the cell type iii) *the nature of the ancillary ligand is* important.

I 512 SYNERGISM BETWEEN AP1 AND NFKBp65-LIKE SITES IS REQUIRED FOR TGF-β1 INDUCTION OF MOUSE TISSUE FACTOR GENE TRANSCRIPTION IN AKR-2B FIBROBLASTS. Sara J. Felts and Michael J. Getz, Biochemistry and Molecular Biology, Mayo Foundation, Rochester MN 55905.
Tissue Factor (TF) is the cell surface receptor for coagulation factor VII/VIIa and is the key control point in the extrinsic pathway for blood coagulation. Interestingly, the gene encoding for TF is rapidly induced in quiescent AKR-2B mouse fibroblasts stimulated with serum or TGF-β1. The kinetics of induction classify Tissue Factor as an "immediate early" gene. In order to identify the DNA elements, transcription factors and cell signaling events responsible for TGF-β1 induction of TF gene expression, various TF promoter sequences were cloned upstream of the chloramphenicol acetyltransferase (CAT) gene of pBLCAT3 for transfection into AKR-2B fibroblasts. Our data show that while induction of TF-CAT by serum, TGF-β1, or even TPA is modest (2- to 3-fold), CAT expression is highly induced (15- to 40-fold) by these stimuli in the presence of protein synthesis inhibitors (cycloheximide, anisomycin, or puromycin). This induction is sensitive to the protein phosphatase inhibitor okadaic acid. Nuclear extracts from TGF-β1-, cycloheximide- or TGF-β1 plus cycloheximide-treated AKRs exhibit an increased AP-1/junB binding activity. Specific binding to an NFKBp65-like motif can also be detected in these extracts. However, this NFKB-like binding does not appear to change significantly with cell stimulation and is of lower affinity compared to consensus NFKB. TGF-β1 induction of TF transcription appears to be mediated, at least in part, by synergistic interactions between factors which bind to two adjacent AP-1 sites and an NFKBp65-like motif.

I 513 COMPLEX INTERACTIONS BETWEEN cAMP, POTASSIUM CHANNEL GENES, AND MEMBERS OF THE CREB/ATF FAMILY OF NUCLEAR PROTEINS, Eduardo Folco, Yasukiyo Mori and Gideon Koren, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115.
We have cloned and expressed two delayed rectifier voltage-gated potassium channels (Kv1.1, Kv1.5) (Koren (1990) Neuron 4, 39), and established that these subunits can form heteromultimers (Matsubara (1991) JBC 266, 13324). Structural analysis revealed that the first transmembrane domain (S1) is critical for the coassembly of heterotetrameric channels, and that the coassembly process depends on the cotranscription and cotranslation of individual subunits (Babila, submitted). Analysis of the transcriptional regulation of Kv1.5 revealed that the half life of the transcript is very short, and that cAMP and depolarization regulate the expression of the channel at the transcriptional level. These effects are conferred by a CRE, located at the 5' noncoding region of Kv1.5 promoter (Mori, JBC, in press). Using published sequences and PCR, we cloned CREB and CREM cDNAs from C2C12 myogenic cell line and rat heart. Analysis of the CREM clones revealed that alternative splicing results in the formation of short variants of CREM that lack the nuclear localization signal, the phosphorylation box and the DNA binding site. These cDNAs code for proteins that are < 10 kDa. RNase protection analysis revealed that in myogenic cell lines cAMP increases the expression of several forms of CREM, a phenomenon that can modulate the transcriptional effects of cAMP. Alternative translation initiation creates a protein that lacks the phosphorylation box and the activation domains but contains the DNA binding site. This form binds Kv1.5 CRE and can form heterodimers with other forms of CREM. Recombinant purified CREM and CREB, phosphorylated in vitro by protein kinase A, were tested for binding of Kv1.5 CRE in electromobility gel shift assays. Phosphorylation results in increasing the binding efficiency, and modifying CREB-CREM interactions. These results indicate that cAMP regulates the expression and function of CREM which in turn may regulate the response of ion channel genes to cAMP and membrane potential. These complex interactions play an important role in controlling cell excitation.

I 514 DIRECT INTERACTION OF THE JUN PROTEIN FAMILY WITH TATA BINDING PROTEIN (TBP) AND TFIIB, Christopher C. Franklin, Vickie A. McCulloch, Clement K. Aseidu, and Andrew S. Kraft, Department of Medicine, Division of Hemaology/Oncology, University of Alabama at Birmingham, B'ham. Al 35294
The Jun family of proteins, c-Jun, JunD and JunB, is capable of binding to upstream DNA sequences, AP-1 sites, and activating transcription. To understand the mechanism of transcriptional activation by the Jun family of proteins, the interaction of these proteins with the TATA box binding protein, TBP, and TFIIB was examined. Protein-protein blot analysis and immobilized fusion protein affinity chormatography were used to study this interaction. Both *in vitro* translated TBP and TFIIB bound to immobilized GST-Jun. By cleaving the TBP cDNA with various restriction enzymes and then translating the protein, a region of TBP spanning only the first direct repeat and the basic region was found to be necessary for c-Jun interaction. Mapping of the TFIIB interaction domain is underway. By *in vitro* translating portions of the c-Jun protein, it was possible to demonstrate that either the amino or carboxy portions of the Jun protein are capable of interaction with TBP. The efficiency of TBP binding to the N-terminal activation domains of c-Jun, JunB, and v-Jun *in vitro* correlated well with their ability to activate transcription *in vivo*. However, although phosphorylation of the c-Jun N-terminal activation domain potentiated c-Jun transcriptional activity *in vivo*, it had no effect on the ability of c-Jun to interact with TBP *in vitro*. Therefore, the Jun family of activator proteins may regulate transcription by directly interacting with the general transcription factors TBP and TFIIB.

I 515 HYPOXIA CAUSES THE ACTIVATION OF NF-κB AND THE PHOSPHORYLATION OF IKBα AT BOTH TYROSINE AND SERINE RESIDUES
Amato J. Giaccia, Eunice Y. Chen, and Albert C. Koong Department of Radiation Oncology, Stanford UniversitySchoolof Medicine Stanford, CA 94305-5468
Nuclear factor κB (NF-κB) regulates the expression of the immunoglobulin light chain genes, the class I major histocompatibility antigen gene, β2-microglobulin, numerous cytokines, and the HIV virus. Transcriptional regulation of gene expression by NF-κB is accomplished by translocation of NF-κB from the cytoplasm to the nucleus where it is able to bind DNA. The signals for NF-κB activation are diverse, but can be classified as protein kinase C (PKC) dependent (*e.g.*, oxidative stress) or PKC independent (*e.g.*., TNF). We reasoned that NF-κB was activated by hypoxia because previous data demonstrated an induction of HIV-1 replication by lowering O_2 tensions. To determine whether NF-κB could be activated by hypoxia to bind DNA and promote transcription of the HIV promotor, we used immunoblot analysis to show an oxygen dependent dissociation of the inhibitory subunit IkBα from NF-κB, and an oxygen dependent increase in p65 nuclear translocation. This increase in nuclear translocation of p65 resulted in increased DNA binding and an oxygen dependent transactivation of a reporter gene construct containing the HIV promotor ligated to the bacterial chloramphenicol acetyl transferase (CAT) gene. Induction of HIV-CAT seemed to be mediated through NF-κB as hypoxia did not induce transcription of the HIV-CAT constructs in which the NF-κB binding sites were mutated. To elucidate the mechanisms of signal transfer from hypoxia to the activation of NF-κB, we studied the protein phosphorylation patterns induced by hypoxia. We found numerous and transitory changes in phosphotyrosine residues, but not in phosphoserine or phosphothreonine residues. These gross alterations in phosphoproteins correlated with the dissociation IkBα from NF-κB during hypoxia. To demonstrate a direct link between changes in phosphorylation patterns of proteins with NF-κB activation, we immunoprecipitated IkBα after varying times of hypoxic exposure and found that both its tyrosine and serine phosphorylation status increased while its threonine phosphorylation status remained unchanged. Furthermore, inhibiting either of these phosphorylation steps was enough to prevent IkBα dissociation from NF-κB and subsequent transactivation of the HIV-CAT reporter construct. These results suggest that *in vivo*, phosphorylation at both tyrosine and serine residues on IkBα are important regulatory steps in the activation of NF-κB by hypoxia.

I 516 HOW IS THE NEUROTROPHIN SIGNAL TRANSDUCED FROM NEURITE TIP TO CELL BODY? Mark Grimes, James Sabry and William C. Mobley, Departments of Neurology and Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0448.

Neurotrophins such as nerve growth factor (NGF) change gene expression and prevent apoptosis in the neuronal cell body by binding to receptors on distant neurites. The nature of retrogradely transported signal is unknown, but could in principle employ any kinase activated by the neurotrophin. PC12 cells are being used to investigate two intracellular organelles that are candidates for the retrogradely transported entity that conveys the NGF signal. The first candidate is an endosome-derived vesicle containing activated gp140^{trk}. Using in vitro reconstitution of membrane traffic and cell fractionation techniques, endosomes and vesicles derived from endosomes can be distinguished based on size, density, and the kinetics with which they acquire labeled NGF or transferrin from the cell surface. The addition of ATP causes the budding of transport vesicles from the early endosome. These vesicles contain NGF and tyrosine phosphorylated gp140^{trk}. These transport vesicles could be recycling vesicles, destined to fuse with the plasma membrane, or vesicles carrying material to the late endosome. If present at neurite tips in neurons, this class may be retrogradely transported. The other candidate signaling organelle is a cytoplasmic particle containing p44$^{ERK1/mapk}$. Velocity and equilibrium density gradient fractionation shows that p44$^{ERK1/mapk}$ particles are slightly smaller, yet more dense than synaptic vesicles. The particles are resistant to detergent lysis and contain at least two other polypeptides whose amount increases upon NGF treatment. In preliminary experiments, microinjection of p44$^{ERK1/mapk}$ particles caused neurite outgrowth in naive PC12 cells. Neurites were observed if donor particles were prepared from NGF-treated cells, but not with donor particles from untreated cells. The data suggest that these particles would be sufficient to induce the NGF response in the neuronal cell body.

I 517 SIGNAL TRANSDUCTION OF INTERLEUKIN-6 (IL-6) IN HUMAN PLASMOCYTOMA CELL LINES INVOLVES ACTIVATION AND TYROSINE PHOSPHORYLATION OF THE SRC-FAMILY KINASE P56^{LYN}, Michael Hallek, Carola Neumann, Brian J. Druker, James D. Griffin, and Bertold Emmerich, Medizinische Klinik, Klinikum Innenstadt, University of Munich, Germany, and Dana Farber Cancer Institute, Boston, USA

Binding of IL-6 to its receptor (IL-6R) induces the association of the IL-6R with gp130, a 130-kDa transmembrane glycoprotein which becomes phosphorylated on tyrosine residues and subsequently transduces a signal to the cytosol. The kinase(s) involved in IL-6 transmembrane signaling has not been identified. In an effort to further characterize the biochemical mechanisms of IL-6 signal transduction, we used two human plasmocytoma cell lines, B9 and LP-1, which are either IL-6 dependent for continuous cell growth (B9) or IL-6 responsive (LP-1). Cells were IL-6 deprived for 18 hrs and then stimulated with recombinant human IL-6. Tyrosine phosphorylation of cytosolic proteins was assessed by SDS-PAGE and subsequent immunoblotting with an anti-phosphotyrosine antibody. In both cell lines, IL-6 induced a rapid, transient and concentration dependent tyrosine phosphorylation of at least five cytosolic phosphoproteins. Maximal effects were observed between 1-15 min after IL-6 stimulation, and at concentrations of 100 ng/mL. Major proteins which were strongly phosphorylated upon stimulation with IL-6, had molecular weights (m.w.) of 80, 160, and 170 kDa (pp80, pp160, pp170), respectively. Minor phosphoproteins had m.w. of 93 and 140 kDa (pp93, pp140). In search of a kinase mediating this tyrosine phosphorylation, immune complex kinase assays with several monospecific antibodies for Src-family proteins were performed. Stimulation with IL-6 consistently induced the activation of p56^{Lyn}, a Src-family kinase expressed in both cell lines. Additional experiments demonstrated that p59^{Lyn} was phosphorylated on tyrosine residues. Taken together, these data suggest that IL-6 induces tyrosine phosphorylation of several cytosolic phosphoproteins via activation of p56^{Lyn}, a tyrosine kinase of the Src-family. These mechanisms may be important for the pathogenesis of plasmocytoma for which IL-6 is an important para- or autocrine growth factor.

I 518 CD40 AND TUMOR NECROSIS FACTOR RECEPTOR p55 (hTNFRp55): A STRUCTURE-FUNCTION-ANALYSIS OF THEIR INTRACYTOPLASMIC DOMAINS, Sigrun Hess, Anne Rensing-Ehl, Gert Riethmüller and Hartmut Engelmann, Institute of Immunology, University of Munich, Goethestr. 31, 80336 Munich, F.R.G.

The human TNFRp55 and Fas antigen (Fas Ag, APO-1) can mediate programmed cell death (apoptosis), whereas B-lymphocyte activation molecule CD40 has been shown to prevent cells from undergoing apoptosis. All three receptors belong to the TNF-NGF-receptor family which is characterized by a four domain extracellular structure with conserved positions of cysteine residues. A second significant homology is found in the intracellular domains (ICDs) of these receptors. This region is essential for intact signal transduction as shown by mutational analysis. In TNFRp55 and Fas Ag this domain is separated from the transmembrane domain by a non-homologous region (NHR) of 137 aa or 55 aa respectively. In contrast CD40, the non-cytotoxic receptor, contains a small NHR of only 9 aa. To analyze whether structurally related parts of TNFRp55 and CD40 are involved in similar functions and non-homologous regions are responsible for differences in function, we studied several hybrid molecules consisting of different portions of both receptors. The extracellular and transmembrane domains of all hybrids consisted of CD40 sequences. The intracellular domain of the first hybrid receptor (hybrid$_1$) consisted of the complete hTNFRp55 ICD. Within this hybrid$_1$ receptor we then exchanged the NHR of TNFRp55 cDNA with the corresponding part (9 aa) of CD40 cDNA (hybrid$_2$). Both hybrids were assayed for their ability to induce cell death after transfection into cell lines sensitive for TNF mediated cytotoxicity (mouse A9, human SV80 fibroblasts). Human CD40 wildtype transfectants served as controls. Stimulation of transfectants with antiCD40 mAb (Laboserv, Giessen, F.R.G.) showed that hybrid$_1$ transduced apoptotic signals in both cell lines, whereas hybrid$_2$ containing the intracellular NHR of CD40 and lacking TNFR NHR was incapable of inducing cell death.

Our data indicate that the non-homologous region of the TNFRp55 is essential for its cytotoxic function.

I 519 PAS MEDIATED PERIOD PROTEIN DIMERIZATION MAY CONTRIBUTE TO THE CIRCADIAN CLOCK MECHANISMS IN DROSOPHILA MELANOGASTER, J. Huang, H. Zheng, K. Curtin and M. Rosbash, Howard Hughes Medical Institute and Department of Biology, Brandeis University, Waltham, Massachussetts 02254

Mutations in the *period* gene product (PER) can shorten or lengthen or eliminate the circadian rhythms of *Drosophila melanogaster*, but its biochemical activity has not been established. PER contains a ca. 270 amino acid motif (termed PAS), also present in three basic Helix-Loop-Helix (bHLH) transcription factors, the *D. melanogaster single-minded gene* product (SIM) and both subunits of the mammalian dioxin receptor complex (ARNT and the AH receptor). We showed recently (Huang et al, 1993) that the PER PAS can function as a novel protein dimerization motif and suggested that PER may regulate circadian gene transcription by interacting with the PAS domain of bHLH-PAS-containing transcription factors. We have now reconstituted these protein-protein interactions in the yeast two hybrid system both for *Drosophila* PER for the two subunits of the mammalian dioxin receptor complex. Consistent with our previous in vitro studies, PER PAS self-associates in yeast. Interestingly, for the two bHLH-PAS proteins of the dioxin receptor, the PAS domain rather than the HLH domain appears to be the major dimerization interface. This yeast system provides a facile genetic approach to structure: function studies of the PAS domain.

The classic *per*^{01} mutation is a missense mutation in the PAS domain which lengthens the circadian period to 29 h and compromises the temperature compensation mechanism of flies' circadian clock. We previously showed that PERL dimerizes poorly in vitro. Here we report that the dimerization efficiency of PERL in yeast is temperature sensitive, decreasing at higher temperatures. Taken together, these observations hint at causal relationship between PAS-mediated dimerization, on the one hand and such circadian clock properties as period length and temperature compensation on the other. Analysis of transformant flies bearing other PAS mutations with dimerization defects is in progress.

Huang, ZH, Edery, I. and Rosbash, M. (1993) Nature 364:259-262

I 520 REGULATION OF TRANSCRIPTION FACTOR E2F BY THE RETINOBLASTOMA PROTEIN: DNA BENDING AS A POTENTIAL MECHANISM, Hans E. Huber, Paula J. Goodhart, Pearl S. Huang, and David C. Heimbrook, Dept. of Cancer Research, Merck Research Laboratories, West Point, PA 19486.

E2F is a mammalian transcription factor involved in cell cycle regulation. The retinoblastoma gene product, pRB, binds to E2F in a cell cycle dependent manner. While this complex formation does not change the sequence specific DNA binding it does appear to turn E2F from a transcriptional activator into a repressor of genes under E2F control, such as myc, polA, and DHFR. We show that *in vitro* binding of pRB has two major effects on the DNA binding properties of E2F which may explain the switch from activator to repressor: pRB binding increases the half-life of E2F-DNA complexes 10 to 15-fold and it partially reverses the DNA bending induced by E2F. Upon specific DNA binding E2F, affinity purified from HeLa cells, induces a DNA bend with a flexure angle of 125^{o}. pRB reduces the apparent DNA bending to less than 80^{o}. DNA footprinting analysis suggests that the non-specific DNA binding activity of pRB is not involved in this effect. The cloned E2F protein, E2F-1, induces DNA flexure similar to HeLa E2F and some DNA bending (50^{o}) is observed with the DNA binding domain of E2F–1 alone. Our biochemical data suggest that transcriptional activation by E2F may involve DNA bending and that the reversal of bending upon binding of pRB may turn E2F into a repressor.

I 521 REGULATION OF THE GENE EXPRESSION BY THE ONCOPROTEIN BCL-3, Jun-ichiro Inoue, Yuzuru Shiio and Tadashi Yamamoto, Department of Oncology, The Institute of Medical Sciences, The University of Tokyo, Tokyo 108, Japan

The *bcl-3* gene was found on chromosome 19 adjacent to the breakpoint in the translocation t(14;19)(q32;q13.1), which occurs in some cases of B-cell chronic lymphocytic leukemia and its expression was demonstrated to be elevated in tumor cells. The product of *bcl-3* gene (Bcl-3) has seven tandem copies of ankyrin repeats through which Bcl-3 binds to p50 and p52 NFκB but not to Rel or RelA(p65). Thus, Bcl-3 is a member of IκB proteins, which regulates transcritional activation mediated by rel family of proteins, but has distinct specificity compared to the other IκB proteins such as IκB α, β and γ. To elucidate the mechanism by which Bcl-3 activates transcription, we have analyzed the effect of Bcl-3 expression on the gene expression from various promoters. The transfection experiments reveal that Bcl-3 activates transcription from the promoter which has no κB-like sequence. These results suggest that Bcl-3 could associate with the protein other than p50 or p52 to activate transcription. Mutational analysis is currently being performed to identify the novel mechanism of transcriptional regulation by Bcl-3.

I 522 CLONING AND CHARACTERIZATION OF A POMC-CRH-RESPONSIVE ELEMENT BINDING PROTEIN IN MOUSE AtT-20 CELLS, WD Jin, AL Boutillier*, M Glucksman, JP Loeffler*, and JL Roberts, Fishberg Research Center for Neurobiology, Mount Sinai School of Medicine, New York, NY 10029, USA; * Institut de Physiologie et de Chimie Biologique, 67084 Strasbourg Cedex, France

A corticotropin releasing hormone (CRH) cAMP-responsive region (-236/-133) has been previously characterized in the POMC 5' regulatory flanking region. This region is composed of several discrete elements that display enhancer-like properties. One novel element designated PPC (POMC palindromic consensus sequence,-171/-160) was most effective (about 3 fold stimulation by CRH) . Gel shift analyses with AtT20 nuclear extracts showed marked enhancement of binding to PPC probe following CRH and forskolin treatment. The binding was inhibited by divalent cations, with Cu^{++} and Cd^{++} being most effective, while Zn^{++} had no effect A protein binding to this element (PPCB) was cloned by Southwestern screening of an AtT20 expression library with radio-labeled PPC oligos. A 2.6 kb cDNA clone (PPCB1) was initially obtained and used as a probe to pull out several other clones to get the sequence corresponding to the entire coding region of the protein. Primer extension and Northern blot analysis revealed that the size of the full length mRNA is about 5 kb.The expression of PPCB mRNA is not restricted to corticotrophs but presents a wide-spread tissue distribution, as evaluated by PCR analysis. Bacterial expressed PPCB1-β-galactosidase fusion protein was shown to bind PPC efficiently by itself. Furthermore, the binding of this fusion protein to the PPC element reveals similar sensitivity to divalent cations as that observed with AtT 20 nulear extracts. Analysis of PPCB protein sequence revealed several intresting motifs: two p-Loop(ATP binding site), several PKA sites in N-terminal regions, and regions of homology to proteins involved in DNA replication and repair. Taken together, PPCB is a potential transacting factor binding to POMC-CRH-resposive element, has interesting characteristics which require further investigation.

I 523 REGULATION OF THE IL-2 RECEPTOR α CHAIN GENE EXPRESSION, S. John, R. Childs, R. Reeves, C. Thompson, and W.J. Leonard, Section on Pulmonary and Molecular Immunology, NIH, NHLBI, Bethesda, MD 20892

IL-2 receptors (IL-2R) critically regulate the magnitude and duration of an immune response and hence are key components of the cascade of events that culminate in a productive immune response. Resting T-cells express intermediate affinity IL-2 receptors which consist of the β and γ chains. The high affinity IL-2 receptor, which is only expressed on activated T-cells, is composed of the α, β, and γ chains. The expression of the α chain of the IL-2 receptor is thus the determining factor in whether or not T-cells express high affinity receptors and thereby its regulation controls the responsiveness of T lymphocytes to IL-2. In this study we have characterized a novel enhancer element between nucleotides -137 and -64 relative to the transcription initiation point of the IL-2R α chain. This enhancer element contains binding sites for at least two major DNA binding proteins; an Ets family member, Elf-1, and the high mobility group protein HMG-I/Y. Deletion or mutation of the binding sites for these proteins results in absent or severely reduced levels of IL-2R α chain gene transcription. Therefore, the protein-DNA interactions that occur at this enhancer region critically regulate the inducible expression of the IL-2R α chain gene and a possible mechanism for how these different factors interact to exert their effects will be presented.

I 524 INVOLVEMENT OF THE ATF/CREB FAMILY IN THE REGULATION OF THE HERPES SIMPLEX VIRUS TYPE-1 (HSV-1) LATENCY-ASSOCIATED TRANSCRIPT (LAT) PROMOTER, Joseph J. Kenny and Brian Wigdahl, The Pennsylvania State University College of Medicine, Hershey, PA, 17033, USA

A latent HSV-1 infection can be reactivated by a variety of extracellular stimuli including stress, fever, iontophoresis of epinephrine into the cornea, and exposure to ultraviolet light. The ability of such extracellular physical and chemical agents to reactivate a latent infection implies that the mechanisms involved in the reactivation process are likely to include neuronal signal transduction pathways. HSV-1 LAT has been implicated in the processes of latency and reactivation. The LAT regulatory sequence (LRS) has been shown to function in a cell type-specific manner. We have constructed an extensive series of deletion mutations of the LRS from nucleotides +1 to -348 to determine specific sequences involved in the cell type-specific activity of the LRS. The series of deletion mutations has been transiently transfected into both mouse neuroblastoma (C1300) and nonneuronal (L929) cells. The first increase in CAT activity detected in any of the cell types examined was observed when sequences from nucleotides -34 to -74 were added to the minimal promoter construct pLRS1-33. The addition of these nucleotides resulted in a similar increase in both neuronal and nonneuronal cell types. This result most likely reflects the addition of the previously described cyclic-AMP response element (CRE) and the LAT promoter binding factor(s) (LPBF) sequence to the minimal promoter. However, the addition of nucleotides -75 to -83 upstream of the LPBF site resulted in a 3- to 4-fold C1300-specific increase in promoter activity. In addition, when sequences were added upstream of nucleotide -178, a second C1300-specific increase in CAT activity was observed corresponding to a second 3- to 4-fold increase in activity. By coupling the functional studies with electrophoretic mobility shift (EMS) analyses of the proximal region of the LRS (nulceotides -54 to -134), antibody supershift EMS, and molecular cloning and analysis of a C1300 cDNA expression library, we have identified the interaction of the ATF/CREB family with nucleotides -75 to -83 of the HSV-1 LRS. The identification of a second ATF/CREB motif in the HSV-1 LRS suggests that the cell type-specific activity of the promoter may involve the interaction of proteins associated with the two ATF/CREB sites. Current studies are directed at delineating the involvement of the CRE sequences in the adenylate cyclase signal transduction pathway, and determining how the factors interacting with these motifs may function collectively in a cell type-specific fashion.

I 525 A SUBSET OF IMMEDIATE EARLY mRNAs INDUCED BY TNF-α DURING CELLULAR CYTOTOXIC AND NON-CYTOTOXIC RESPONSES, Juha Klefstrom, Päivi J. Koskinen, Eero Saksela, Marja Jäättelä*, Rodrigo Bravo#, and Kari Alitalo, Molecular/Cancer Biology Laboratory, Department of Pathology, P.O. Box 21 SF-00014 University of Helsinki, Finland; Department of Pathology*, The University of Michigan Medical School, Ann Arbor, MI 48109; Department of Molecular Biology#, The Squibb Institute for Medical Research, P.O.BOX 4000, Princeton, NJ, USA.

TNF-α is a multifunctional cytokine which is cytotoxic for many tumor cell lines. In order to characterize the early genomic response to TNF-α, we have analysed the induction of a subset of serum-inducible immediate early genes in WEHI-S and L929 fibrosarcoma cell lines, which are sensitive to TNF-α and in the 3T3-L1 pre-adipocytic cell line, which is resistant to TNF-α cytotoxicity. Among 77 immediate early mRNAs screened by dot blot and/or Northern blot analyses, the expression of 23 mRNAs was found to be induced by TNF-α. Ten of these mRNAs encode proteins known to function as proinflammatory cytokines or transcription factors, while thirteen others have as yet uncharacterized activities. The magnitude of *c-fos* induction by TNF-α inversely correlated with cell type-specific cytotoxicity. Rapid and transient mRNA responses were observed in the TNF-α-resistant cells, whereas a slower and more persistent response was characteristic for TNF-α-sensitive cells. The prolonged induction of immediate early mRNAs may contribute to TNF-α-induced cellular cytotoxic responses. The relation of these changes to TNF induced cytotoxicity have been assessed by overexpressing certain of these genes from inducible constructs.

I 526 TOR1 AND TOR2 ARE HIGHLY RELATED PHOSPHATIDYLINOSITOL KINASE HOMOLOGS INVOLVED IN RAPAMYCIN TOXICITY AND G1 PROGRESSION IN *SACCHAROMYCES CEREVISIAE*, Jeannette Kunz, Ulrich Schneider, Stephen B. Helliwell, Nik Barbet, Maja Deuter-Reinhard, and Michael N. Hall, Department of Biochemistry, Biocenter, University of Basel, CH-4056 Basel, Switzerland

The immunosuppressive drug rapamycin causes G1 arrest in activated T cells and yeast. Three genes, *FPR1*, *TOR1* and *TOR2*, have been identified in *S. cerevisiae* by selecting spontaneous rapamycin resistant mutants. *FPR1* encodes the cytoplasmic receptor of rapamycin which mediates drug action and is a homolog of the human proline isomerase FKBP12. *TOR1* and *TOR2* encode a pair of large (282kDa) and highly related (66% amino acid identity) proteins which contain a domain homologous to the catalytic subunit p110 of bovine phosphatidylinositol 3-kinase (PI 3-kinase) and to the yeast PI 3-kinase VPS34. We have termed this domain the lipid kinase domain. *TOR2* disruption is lethal, but does not result in a cell cycle-related arrest phenotype. *TOR1* disruption is not lethal, but, when combined with a *TOR2* disruption, causes cells to arrest in G1, as does exposure to rapamycin. These findings suggest that *TOR1* and *TOR2* possess an overlapping, essential cell cycle-related function, whereas *TOR2* fulfills an additional essential function. *TOR1-TOR2* hybrids demonstrate that the shared function is mediated by the lipid kinase domain. Our results suggest that TOR1 and TOR2 are the targets of drug action in yeast and that rapamycin-FKBP12 acts by inhibiting TOR PI kinase activity required for G1 progression.

Work in progress should identify other components in the rapamycin-sensitive signaling pathway which may play an important role in cell cycle regulation in yeast.

I 527 PITSLRE 1 KINASE ACTIVITY INDUCES APOPTOSIS, Jill M. Lahti[1], Jialing Xiang[1,2], Lucie Heath[3], and Vincent Kidd[1], [1]Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN 38105, and Departments of [2]Cell Biology and [3]Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294

Introduction of a gene encoding a $p34^{cdc2}$-related protein kinase, $p58^{GTA}$ (renamed PITSLRE 1) results in late-telophase delay and abnormal cytokinesis and leads to apoptosis in CHO fibroblasts. Programmed cell death occurs even in cells expressing enzymatically active PITSLRE 1 mutants that no longer induce cell cycle or mitotic abnormalities, but not in cells expressing enzymatically inactive forms. Overexpression of either $p34^{cdc2}$ or other atypical cdc2 family members does not result in apoptosis in these cells. Additional studies with rat myeloid 32D cells, which are uncommitted precursors dependent on IL-3 for survival, support this hypothesis. Withdrawal of IL-3 leads to apoptosis, and cells cannot be rescued after ~4-6 hrs. We find that PITSLRE kinase activity is induced at least 50-fold in these cells 2-3 hrs. after IL-3 withdrawal. These results suggest that the PITSLRE 1 protein kinase is linked to an apoptotic signalling pathway.

I 528 A TRUNCATED CYCLIN D1 TRANSCRIPT IS OVEREXPRESSED IN A HUMAN BREAST CANCER CELL LINE BEARING AN ALTERED D1 GENE,
David E. Lebwohl, Robin Muise-Helmericks, Laura Sepp-Lorenzino, Susanne Serve, Patrick Borgen, Neal Rosen, Departments of Medicine and Surgery, and Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

The G1 cyclin PRAD1, or cyclin D1, gene is rearranged and overexpressed in human parathyroid tumors. These findings imply that increased expression of cyclin D1 allows it to act as an oncogene in the parathyroid tumors. PRAD1 is closely linked and may be allelic to the BCL-1 locus at 11q13, which was originally identified by its translocation to an immunoglobulin gene enhancer in some B cell leukemias. Of note, BCL-1 and cyclin D1 are amplified in approximately 20% of human breast cancers, and cyclin D1 is frequently overexpressed in these tumors. Cyclin D1, then, has a potential role in the pathogenesis of breast cancer via amplification of the gene and overexpression of this regulatory protein. Although amplification of cyclin D1 in human breast cancer may be important in carcinogenesis, another gene in the amplicon may be acting as a dominant oncogene in these cancers. However, we now report a truncation of the cyclin D1 gene in a human breast cancer cell line, which also shows overexpression of a short cyclin D1 message. In a survey of breast cancer cell lines and tumors by Southern blot hybridization, using a 1.15 kB human cyclin D1 cDNA probe, we found that the blot of MDA MB-453 cell line DNA contained an extra band in the BglII digest, suggesting that one allele of gene was altered. Moreover, the altered allele was amplified three fold relative to the normal allele(s). The alteration was shown to be a 3' deletion, based on Southern blotting with a probe containing only 3' untranslated sequence. This showed no hybridization to the altered band in 453, implying that this sequence is deleted in the altered gene. On Northern analysis, the MDA MB 453 line has a marked increase in transcripts in the 1.1-1.3 kB range relative to the normally predominant 4.7 kB transcript. This mRNA is truncated at the 3' end, as demonstrated by a probe from the 3' untranslated region of cyclin D1 and by RNase protection. These alterations in the cyclin D1 gene and mRNA suggest that activation of cyclin D1 may be important in the malignant transformation of this cell line, and support the identification of cyclin D1 as an oncogene amplified in human breast cancer.

I 529 CHARACTERIZATION OF EARLY INDUCED GENES OF TGF-β DURING G1 PHASE OF CELL CYCLE
Mong Hong Lee and Joan Massagué, Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center and Howard Hughes Medical Institute, 1275 York Ave. Box 116, New York, NY 10021

Transforming growth factor-beta (TGF-β) mediates G1 cell cycle arrest through a mechanism that involves inhibition of cyclin E-dependent kinase activity and suppression of Rb phosphorylation. The pathway linking receptor signals to the growth inhibitory events are not known. However, we found that various transcriptional inhibitors interfere with the activity of TGF-β to prevent Rb phosphorylation, suggesting the involvement of TGF-β early response genes in the growth inhibitory response. In order to identify such genes, we are using a differential display method to amplify the cDNAs corresponding to genes that are specifically induced by TGF-β in epithelial cells during G1. By comparing the cDNAs amplified by PCR from TGF-β treated and non-treated mink lung epithelial cells, we are attempting to identify the inducible genes of interest. Twenty seven clones isolated so far represent candidate of TGF-β early response genes are in the process of characterization. This information shall shed light on the molecular mechanism of antiproliferative TGF-β action.

I 530 Role of Redox-Sensitive Signals in the Induction of E-Selectin, VCAM-1 and ICAM-1 Gene Expression.
Anthony M. Manning, Cecil C. Chen, Alistair R. McNab, Craig L. Rosenbloom and Donald C. Anderson. Adhesion Biology, Upjohn Laboratories, Kalamazoo, MI 49001.

We have tested the hypothesis that antioxidant-sensitive signals act as second messengers mediating the induction of cell adhesion molecule gene expression in endothelial cells. Promotor analyses has demonstrated that the redox-sensitive nuclear transcription factor NFκB is essential for the cytokine induction of expression of the E-selectin, VCAM-1 and ICAM-1 genes. We demonstrate that antioxidants inhibit the cytokine-induced activation of the nuclear transcription factor NFκB in endothelial cells and also inhibit the induction of transcription of the cell adhesion molecule genes E-selectin, VCAM-1 and ICAM-1. Three structurally distinct antioxidants, N-acetylcysteine (NAC), pyrrolidine dithiocarbamate (PDTC) and bunaprolast (U-66858), inhibited the cytokine-induced activation of NFκB in human umbilical vein endothelial cells (HUVEC). DNA-binding activity of the transcription factors AP-1, SP-1 and CREB were unaffected. These antioxidants also significantly inhibited the cytokine-induced elevation of E-selectin, VCAM-1 and ICAM-1 mRNA levels, but did not affect mRNA levels for ICAM-2, a constitutively expressed CAM whose expression is not cytokine-responsive. The TNFα-induced activity of an E-selectin promotor-luciferase reporter construct was inhibited in a dose-dependent manner by PDTC and NAC both in the endothelial cell line EAhy926 and in Hela cells. The antioxidants also inhibited, in a dose-dependent manner, the upregulation of cell surface expression of E-selectin, VCAM-1 and ICAM-1 protein following stimulation with either tumour necrosis factor α (TNFα), interleukin 1β (IL-1β), or bacterial lipopolysaccharide (LPS). Expression of ICAM-2 was not significantly affected, indicating that the effect of these compounds were specific. These data demonstrate that redox-sensitive signals play an important role as intracellular signals in the induction of cell adhesion molecule gene expression in human endothelial cells.

I 531 INVOLVEMENT OF AN INTERFERON-STIMULATED 91kDa TRANSCRIPTION FACTOR IN THE REGULATION OF *C-FOS* BY GROWTH HORMONE,
Debra J. Meyer, George S. Campbell, Brent H. Cochran*, Andrew C. Larner†, Christin Carter-Su, Jessica Schwartz, Dept. of Physiology, U. of Mich., Ann Arbor, MI, *Dept. of Physiology, Tufts U. Med. School, Boston, MA, †Ctr. Biol. Eval. Res., Bethesda, MD.

Growth hormone (GH), a major regulator of normal growth, induces *c-fos* transcription. However, signalling mechanisms leading to regulation of gene transcription by GH have been elusive. To identify GH-inducible DNA binding complexes, the *c-sis* inducible element (SIE) of the *c-fos* promoter was tested in mobility shift assays with nuclear extracts from 3T3-F442A fibroblasts, which differentiate into adipocytes in response to GH. A growth hormone-inducible DNA binding factor (GHIF) was identified which is rapidly induced by GH at physiological concentrations. Since recent findings indicate that GH and other members of the cytokine receptor family, including interferons (IFNs), activate JAK family tyrosine kinases, we evaluated whether GHIF might contain components related to DNA binding complexes that are activated by IFN α or IFN γ. Antibodies to p91, one component in IFN-induced complexes, recognized the GHIF complexes, causing a supershift. Similar to p91, GHIF also contains phosphorylated tyrosine. Furthermore, GH stimulates tyrosyl phosphorylation of cellular proteins antigenically related to p91. These findings indicate that GH inducible DNA binding proteins contain p91-related proteins, and suggest that in signalling between their receptors and the nucleus, GH and IFNs utilize related or identical components, including JAK family tyrosine kinases and proteins in the p91 family.

I 532 THE OVEREXPRESSION OF HUMAN HEAT SHOCK TRANSCRIPTION FACTOR -1 DOES NOT RESULT IN AN INCREASE IN THE HEAT SHOCK PROTEIN SYNTHESIS IN MURINE CELLS, Nahid F. Mivechi, Xaio- Y. Shi, George, M. Hahn, Department of Radiation Oncology, Stanford University School of Medicine, Stanford, CA 94305.

Transcription of heat shock genes is regulated by the activation of the heat shock transcription factor (HSF). After heat shock, HSF-1 forms a trimer and binds to the heat shock element (HSE) which consists of 3 repeats of NGAAN located in an inverted orientation in the promoter region of the heat shock genes. HSF-1 is then phosphorylated activating transcription of the heat shock genes likely by transactivation. We have overexpressed the human heat shock transcription factor -1(HSF-1) in murine cells to study its properties and to find out if HSF-1 overexpression leads to the enhanced synthesis of heat shock mRNAs after heat shock. HSF-1 cDNA was cloned into a retroviral vector (pvhhsf-1) and was overexpressed in a murine fibroblast cell line. The overexpressed human HSF-1 is found in the cytoplasm and nucleus of control cells and is translocated into the nucleus upon heat shock. The overexpressed HSF-1 has constitutive DNA binding activity, although it is not transcriptionally active. Western blot analysis shows that human HSF-1 is phosphorylated and shifts in molecular weight by approximately 10 kDa following heat shock. Further, cross linking experiments show that the overexpressed HSF-1 is in the monomeric form and trimerizes upon heat shock. In spite of both the DNA binding ability and phosphorylation of transfected HSF-1 after heat shock, the human HSF-1 does not increase the transcription of murine HSP-70 mRNA or protein after heat shock beyond that observed in control untransfected cells. Cells overexpressing HSF-1 are 10 fold more resistant to the cytotoxic effects of heat compare to control untransfected cells. However, the kinetics of thermotolerance development and decay is the same as that observed in control cells. The transfected cells grow more slowly; specifically longer G1 phase of the cell cycle is observed. In conclusion, overexpression of human HSF-1 protein does not result in overproduction of heat shock proteins in cells containing abundant amount of endogenous HSF-1. Other characteristics of these cells will be further presented.

I 533 PHEROMONE-INDUCED G_1 ARREST IN *S. Pombe*, Olaf Nielsen and John Davey[1], Department of Genetics, University of Copenhagen, DK-1353 Copenhagen K, Denmark and [1)] School of Biochemistry, University of Birmingham, B15 2TT England

In the fission yeast *S. pombe* pheromone communication and mating is activated by nitrogen starvation - a condition which also causes arrest in the G_1 phase of the cell cycle. Two different classes of mutants have been described, which are derepressed for mating in nitrogen containing media. These are either defective in the *pat1* protein kinase or have reduced levels of cAMP. Here we report that when mitotically growing *P* cells belonging to either of these classes are exposed to *M* factor pheromone, they arrest in G_1, and induce transcription of the pheromone controlled *mat1-Pm* gene. After approximately two generation times, the cells overcome the arrest and re-enter the mitotic cycle, suggesting that mechanisms of desensitization exist. These findings suggest that *S. pombe* - like *S. cerevisiae* - can block the transition from G_1 to S in response to pheromone signal transduction.

I 534 TRANSCRIPTION FACTORS IN MYELOID-RESTRICTED AND IFN-γ INDUCED GENE EXPRESSION, Christophe Perez, Eliane Coeffier, Juana Wietzerbin and Philippe D. Benech. Unité 365 INSERM, Institut Curie, Section de Biologie, 26 rue d'Ulm, 75231 Paris cédex 05, France

Expression of the gene encoding the human high affinity receptor for IgG (hu-FcγRI) depends on two distinct cis-DNA elements. One element, termed GRR is involved in the gamma interferon (IFN-γ) activation of the hu-FcγRI gene expression. Downstream to this sequence, another element, called MATE, is responsible for the myeloid restricted expression of this gene. Both elements, GRR and MATE are the targets for distinct multiproteic complexes, termed respectively GIRE-BP and MATE-BP.

GIRE-BP is activated upon IFN-γ /-α treatment in hematopoietic and non hematopoietic cell lines. Although the GRR motif is not related to the interferon responsive elements (ISRE and GAS), GIRE-BP shares the 48- and the 91-kDa proteins of the IFN-α activated complex, ISGF3. Specific phosphorylation of the 91-KDa protein would occur after activation of an IFN-γ-dependent kinase different from the recently identified IFN-α-dependent kinase (tyk-2). In addition to the 48 and 91 kDa proteins, GIRE-BP required another protein that we called the *Link* factor which provides the DNA binding site recognizing the GRR motif.

MATE-BP activity was detected only in myeloid and B cell extracts as two different electrophoretic mobility complexes. Formation of these complexes did not depend on IFN activation but was reduced by PMA and IL4 treatment. Both complexes shared with GIRE-BP, the link factor. The fact that MATE-BP activities were cell-type specific suggested that these complexes requires in addition to the *link* factor, the presence of a protein absent in non-hematopoietic cells. Experiments performed in order to identify the factors involved in GIRE-BP and MATE-BP led presently, to the cloning of one cDNA encoding the myeloid and B cell transcription factor, PU.1.

Antibodies raised against PU.1 abolished the formation of the two MATE-BP complexes.

Therefore, we concluded that myeloid restricted and IFN-γ induced expression of the hu-FcγR1 gene required respectively two types of protein interactions: one will depend on the association of the *link* factor with PU.1 to form the MATE-BP complexes, the second will result in a combination of the *link* factor with the 48 Kda and the IFN-γ dependent phosphorylated 91 KDa protein to ellicit the IFN-γ activated GIRE-BP complex.

I 535 TNF RECEPTORS TR60 AND TR80 MEDIATE APOPTOSIS VIA INDUCTION OF DISTINCT SIGNAL PATHWAYS, Klaus Pfizenmaier, Peter Scheurich, Gudrun Zimmermann, Beate Maxeiner and Matthias Grell, Institute of Cell Biology and Immunology, University of Stuttgart, 70569 Stuttgart, Germany

Tumor necrosis factor receptors are usually coexpressed in many tissues, but their relative contribution to cellular TNF responses is, for most situations, unknown. In a TNF cytotoxicity model of KYM1, a human rhabdomyosarcoma cell line, we could recently show that both TNF receptors independently induce cell death (Grell et al., 1993, Lymphokine and Cytokine Research 12, 143). Interestingly, both receptors activate signals resulting in apoptosis with a similar rapid onset of DNA fragmentation and the typical morphological signs. Variants of KYM1 with increased resistance to TNF mediated cell death revealed that sensitivity can be controlled at both the receptor and a post receptor level. In the latter case resistance is selective for a given TNF receptor type; i.e. cells resistant to TR60 mediated cytotoxicity remain sensitive to treatment with TR80 specific agonistic antibodies. To identify signal pathways involved in TR60 and TR80 induced apoptosis, we have employed a series of selective inhibitors of intracellular signalling molecules. The observed differential sensitivity to these inhibitors provide first evidence for a distinct signal pathway usage of TR60 and TR80, involving a PLC / PK pathway and a arachidonic acid / lipoxigenase pathway, respectively.

I 536 CYTOPLASMIC DEGRADATION OF *FOS* AND *JUN* FAMILY PROTEINS BY CALCIPROTEASES.

Marc PIECHACZYK, Magali, PARIAT, Ann-Muriel STEFF, Isabelle JARIEL-ENCONTRE, Pierre ROUX and Serge CARILLO.*IGMM/UMR9942, CNRS, BP5051, Route de Mende, 34033, Montpellier Cedex 01, France.*

AP-1 transcription factor is crucial for transforming short-term stimulations into long-term responses of varied nature. It is a complex of different proteins including those of the *FOS* and *JUN* family which bind DNA. Most of the latter are known to display a short nuclear turn-over which contributes to the rapid gene expression down-regulation. It has not yet been formally demonstrated whether this corresponds to rapid degradation within the nucleus or to return into the cytoplasm (although the former hypothesis is plausible because of the presence of proteolytic activities within the nucleus). While investigating the control of *c-FOS* (Roux et al., Cell 63, 341, 1990) and *c-JUN* (Roux et al., the Fos oncogene, Angel and Herrlich, Eds, CRL Press, 1993) protein transport into the nucleus, we have shown that *c-FOS* is highly unstable in the cytoplasm and thus pointed to the existence of a new regulation of the nuclear steady-state level through the control of the amount of full-length molecules available for nuclear transport. Using cytoplasmic extracts from various origins, we now report that : (i) *c-FOS* degradation can be initiated in a calcium-dependent, ATP-independent manner which involves cysteine proteases called milli- and micro-calpain; (ii) interestingly, *FOS-B*, a member of the *fos* multigene family, as well as all members of the *jun* family (*JUN-B*, *c-JUN* and *JUN-D*) are also sensitive to calpains albeit to different extents, (iii) *FRA-2*, which is a *c-FOS*-related protein, is resistant to micro- but not to milli-calpain whereas FRA-1, another member of the *fos* family, is resistant to both proteases, (iv) *FOS-JUN* interaction is not necessary for rapid calpain-mediated degradation. Our observations thus point to the existence of (i) a novel specific contribution to the regulation of AP-1 transcription complex activity through the differential control of the steady-state level of some of its components (ii) alternate degradation mechanisms operating on *c-FOS* since, using another cell-free degradation assay, others (Papavassiliou et al., Science 258,1951, 1992) have characterized an ATP- and phosphorylated *JUN*-dependent catabolism pathway of *c-FOS*. Interestingly, of the mutated *FOS* proteins transduced by the osteosarcomatogenic murine retroviruses FBJ-MSV and FBR-MSV, *v-FOS^{FBR}* is resistant to calpains. This likely contributes to the accumulation of this protein to high levels in infected cells and therefore to the higher tumorigenic potential of FBR-MSV.

I 537 MUTATIONS IN *LOZENGE* AFFECT THE SELECTION OF CELL TYPE IN THE DEVELOPING DROSOPHILA EYE.

J. A. Pollock*, P. Batterham†, P. Maurides*, C. Nichols*, A. M. Sokac* and J.R. Crew*. †Department of Genetics, The University of Melbourne, Parkville, Victoria 3052, Australia; *The Center for Light Microscopic Imaging and Biotechnology and the Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213.

In Drosophila, as in other organisms, it has been found that cell to cell communication through transmembrane signal transduction governs complex differentiation of neurons as well as the neural networks responsible for vision. For the fly, differentiating photoreceptor neurons communicate with one another to establish specific fates. Once this has happened, axons from the developing retina are directly responsible for the induction of their neural targets in the brain. Cells that are unnecessary for normal adult function receive programmed instructions to die through apoptosis. In our study of the basic biology of these events, we have found that a gene called *lozenge* may affect these processes.

Mutations in *lozenge* cause a pleiotropic range of phenotypes including severe morphological defects in the adult sensory organs for taste, smell and sight. The *lozenge* gene appears to function in the regulation of cell fate selection at more than one determinative step in the development of these organs. Our developmental and genetic study of *lozenge* mutant eyes indicates that defects begin early in pupal development. We are studying the *lozenge* phenotypes with various microscopy techniques. This is permitting the identification of specific cell types that are affected by *lozenge*. Furthermore, we have found altered patterns of programmed cell death occurring in mutant alleles, the severity of which correlates with the defects apparent in the eye and brain. Staining the developing retina with acridine orange reveals a synchronous band of dying cells. Electron microscopy confirms pynotic nuclei in these strains. We have also found that certain *lz*$^+$ strains have a similar pattern of acridine orange stained cells in the developing eye; these strains have nearly normal eyes. We are investigating this non-*lozenge* associated cell death to resolve whether it is associated with a genetically identifiable locus on the first chromosome. A model for the normal cellular interactions that occur during eye development will be discussed in light of the *lozenge* eye phenotype.

The molecular cloning of *lozenge* is underway in our laboratory; results of this work-in-progress will be presented as well.

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I 538 ANALYSIS OF REPORTER TRANSGENIC MICE FOR AP-1 ACTIVITY

Mercedes Rincón and Richard A.Flavell. Section of Immunobiology, Yale University School of Medicine, New Haven, CT 06511.

Activation of T cells induce transcription of the IL-2 gene. IL-2 expression is regulated through the binding of transcription factors to multiple sites within the IL-2 enhancer, such as NF-kB, NFAT, AP-3, Oct-1 and AP-1. The transcription factor designated AP-1 is a complex mixture of homodimers and heterodimers containing various members of the Fos and Jun families of proteins. At least five members of the Fos family protein (c-Fos, FosB, FosB2, Fra-1 and Fra-2) and three members of the Jun family (c-Jun, Jun B and Jun D) have been identified. So far, most of the prior studies related to AP-1 in primary T cells have been focused on the DNA binding activity of this complex, using the electrophoretic mobility shift assay. In order to analyze the transactivation function of the AP-1 complex during development and activation of T cells, we have generated reporter transgenic mice. These mice have integrated in their genome the luciferase gene driven by two AP-1 DNA binding sites in the context of the minimal rat prolactin promoter. Resting cells from spleen, lymph nodes, and thymus from transgenic mice do not express luciferase activity. However, a significant luciferase activity is observed upon activation with PMA plus Ionomycin. The luciferase activity is transient, a result that correlates with AP-1 DNA binding activity previously described. Similar results but different kinetic profiles have been obtained using specific stimuli such as anti-CD3 or anti-TcR monoclonal antibodies or Concanavalin A in splenocytes. We are currently analyzing the AP-1 transcriptional activity in different stages of thymus development and the signalling requirements in T cell activation. It would be interesting to compare the previous DNA binding activity data with the transactivation function of the AP-1 complex in primary culture.

I 539 PHYSIOLOGICAL REGULATION OF C-*FOS* EXPRESSION REQUIRES MULTIPLE INTERDEPENDENT TRANSCRIPTION CONTROL ELEMENTS,

Linda M. Robertson, Tom Kerppola, Montserrat Vendrell, Daniel Luk, Richard J. Smeyne, James I. Morgan, and Tom Curran, Roche Institute of Molecular Biology, Nutley, NJ 07110

The protooncogenes, c-*fos* and c-*jun*, are cellular immediate-early genes that are induced in response to a variety of extracellular stimuli. Their protein products (Fos and Jun) form heterodimers that regulate transcription of target genes containing AP-1 and CRE binding sites. We have developed a transgenic mouse line containing a c-*fos*lacZ fusion gene. To characterize the molecular basis of c-*fos* regulation *in vivo*, we also produced transgenic mice carrying a c-*fos*lacZ gene containing mutations in the known regulatory elements. Constituitive expression of the intact transgene has been observed in a number of locations including bone, skin, and hair cells. In marked contrast, little or no expression was detected in these locations in any of the mutant transgenic strains studied. Mutations in the v-*sis* inducible element (SIE), the serum response element (SRE), the c-*fos* AP-1 site, and the Ca/cAMP response element (CRE) each eliminated virtually all of the constituitive c-fos expression at these sites. In addition to constituitive expression, the intact fos-lacZ transgene can be induced by a variety of stimuli. For example, injection with the seizure-inducing agent, kainic acid results in high levels of transgene expression in the central nervous system. This characteristic pattern of expression was dramatically altered in all the mutant lines analyzed. Previous studies of gene regulation have relied heavily on the use of cultured cells. To compare the expression of transgenes in the intact animal with their expression in cell culture, primary fibroblasts were prepared from each transgenic line and ß-gal activity was monitored in response to a variety of stimuli. Exposure to high serum, TPA, PDGF, and cAMP increased expression of ß-gal activity in fibroblasts from transgenic lines containing the intact c-*fos*lacZ gene. Fibroblast cultures from trangenic animals that carrried transgenes with mutations in the SIE, SRE, or FAP exhibited little or no response to any of the agonists tested. This is in contrast to the prediction that these elements could function independently to mediate responses to specfic signals in cultured cells Taken together, these *in vivo* and *in vitro* results suggest that the regulation of c-fos expression in a physiological context requires the interdependent function of all the regulatory elements tested.

I 540 A RECOMBINANT SOLUBLE RECEPTOR FOR IgG INHIBITS PROGRESSION OF ACTIVATED B CELLS IN THE G1 PHASE OF THE CELL CYCLE, Catherine Sautès, Caroline Bouchard, Daniel Choquet ° and Wolf-Herman Fridman, INSERM U255, Institut Curie, 26 rue d'Ulm, 75005 Paris and ° INSERM U261 Institut Pasteur, 75015 Paris, France.

Antibody production is the consequence of a series of interactions which drive B cells through activation, proliferation and differentiation. Under soluble forms, low affinity receptors for the Fc portion of IgG (FcγR) have been shown to down-regulate antibody production. We have produced in eukaryotic cells and purified to homogeneity a recombinant soluble FcγR (sFcγR) corresponding to the external region of mouse low affinity FcγRII. We show that it inhibits proliferation -as measured by [^{3}H] Thymidine incorporation- of mouse resting B lymphocytes stimulated by LPS or by Fab'2 fragments of anti-IgM. sFcγR has no significant effect on the early activation events occuring after anti-IgM stimulation, such as the phosphorylation of proteins on tyrosine residues and the transient rise of intracellular calcium -measured at the single cell level- neither has it any effect on the induction of *c-myc* mRNA detectable 45 min and 3 hr after anti-IgM or LPS stimulation respectively.

The entry of B cells in cell cycle was analyzed by measuring cell size and expression of cell surface markers of the G_0 and G_1 phases. sFcγR does not affect the increase of expression of class II antigens which occurs before the activated B cells enter G_1. However it inhibits cell enlargment as well as the induction of Transferrin receptors, a marker of the late G_1 phase. Thus, sFcγR inhibits B cell proliferation by blocking events of the late G1 phase. Analysis of its effect on the expression of G1 phase-specific cyclins and on phosphorylation of the retinoblastoma gene product Rb are under investigation.

I 541 CLONING AND EXPRESSION OF A LYMPHOID-SPECIFIC MEMBER OF THE G-PROTEIN COUPLED RECEPTOR KINASE FAMILY

David Chantry, Byron Sebastian, Patrick Gray. ICOS Corporation, 22021 20th Avenue SE, Bothell, WA 98021. FAX 206-485-1961.

The cloning of receptors for both classical chemoattractants (C5a, FMLP) and a growing number of chemokines (IL-8, GRO, RANTES) has shown that they belong to the group of receptors which are coupled to heterotrimeric G proteins. A common feature of this family of proteins is that transient exposure to ligand leads to a loss of subsequent responsiveness, a process termed homologous desensitisation. In the case of the rhodopsin and the β-adrenergic receptors desensitisation is dependent on phosphorylation of the ligand-occupied receptor. The kinases responsible for the desensitisation of rhodopsin and the β-adrenergic receptor have been identified and shown to be structurally and functionally related proteins. As an initial approach to understanding the molecular mechanism of desensitisation of the chemoattractant receptors we have used degenerate PCR to look for lymphoid specific members of this family.

We have cloned a novel protein kinase which we have called GRK-6 (for G-protein coupled receptor kinase). Sequence analysis of GRK-6 shows that it is part of a family of acidophillic protein kinases which includes rhodopsin kinase and the β-adrenergic receptor kinase. A preliminary analysis of the *in vivo* expression of this kinase by northern blotting indicates that it is most abundant in lymphoid tissues (thymus and tonsil), where a ~3 kb mRNA is seen; but is not detectable in a panel of other tissues (placenta, heart, brain, liver and kidney). Expression has also been detected in a range of lymphoid and myeloid cell lines. The catalytic domain of GRK-6 has been engineered for expression in *E.coli* as a fusion protein with thioredoxin, and we have demonstrated that our cDNA clone encodes a functional protein kinase, which when over-expressed in *E. coli* is capable of phosphorylating a range of endogenous proteins. In view of its restricted tissue distribution and structural similarity to other GRKs, GRK 6 may regulate signal tranduction through lymphoid specific G protein coupled receptors. Work is in progress to identify the physiological substrate(s) for this kinase.

I 542 THE INVOLVEMENT OF MAP KINASES ERK1 AND ERK2 IN THE REGULATION OF C-FOS EXPRESSION BY TRANSFORMING ONCOGENES AND GROWTH FACTORS Peter E. Shaw, Hendrik Gille, Monika Kortenjann and Oliver Thomae. Max-Planck-Institut für Immunbiologie, Stübeweg 51, 79108 Freiburg, Germany

Transcription of proto-oncogene c-*fos* is stimulated rapidly and transiently by a variety of serum growth factors and mitogens. Critical for this response is the serum response element (SRE), which is known to be bound *in vivo* by the transcription factors SRF and TCF/Elk-1.

It has been shown that disruption of TCF/Elk-1 binding by mutations in the SRE correlates with impaired induction by serum and phorbol esters and that phosphorylation of TCF/Elk-1 by MAP kinases is one step in the stimulation of c-*fos* transcription through the SRE.

We have begun to characterise the role of MAP kinases in the activation of c-*fos* expression by transforming oncogenes. Transactivation of c-*fos* expression by oncogenic forms of Raf-1 kinase requires the SRE and can be attenuated by interfering mutants of MAP kinases erk1 and erk2. Replacement of the "*ets*" DNA binding domain of TCF/Elk-1 with that of Gal4 produces a hybrid protein (Gal-Elk) that can mediate transactivation of a Gal4 reporter by v-*raf*. The transactivation is abrogated by mutation of a single serine residue in the C-terminal domain of Gal-Elk that is a major site of phosphorylation by MAP kinases *in vitro* and is known to be phosphorylated in Elk-1 *in vivo*. These results indicate that the mechanism by which v-*raf* transactivates c-*fos* expression involves phosphorylation of TCF/Elk-1 by activated MAP kinases and that this may represent an essential component of the transformation process by oncogenic forms of Raf-1 kinase.

I 543 THE ROLE OF RAPAMYCIN-SENSITIVE INTRACELLULAR SIGNALLING PATHWAYS IN T AND B LYMPHOID CELL CYCLING.

Malcolm C. Smith, G. Yvonne Morgan, Donna M. Osborn & John E. Kay. School of Biological Sciences, University of Sussex, Brighton BN1 9QG

Rapamycin has been shown to block the phosphorylation and activation of the 70K S6 kinase in mitogen-activated mammalian cells., Inhibition is not direct, but is effective whether the activation cascade is initiated via receptor-linked tyrosine kinase activation or phorbol ester activation of protein kinase C. This suggests that the direct target of rapamycin inhibition is an upstream regulator common to both 70K S6 kinase activation pathways.

The rapamycin sensitivity of proliferation of different B and T lymphoid cell lines varies. Some cell lines appear resistant to its action. In several B and T cell lines cells reactivated from the stationary phase of the cell cycle were much more sensitive to rapamycin than cells maintained in continuous log phase. Hypersensitivity to rapamycin, with a cytotoxic rather than cytostatic response to particular concentrations, was seen in one B cell line (BJAB). Proliferation of these cell lines was not sensitive to 100 nM FK506, but rapamycin inhibition was substantially reversed by excess FK506. This indicates that rapamycin inhibition of proliferation is mediated through blockade of a mitogen-activated signalling pathway (rather than simple inhibition of the peptidilprolyl isomerase activity of a rapamycin- and FK506-binding FKBP) involving a PPIase that binds both FK506 and rapamycin (probably FKBP-12). Rapamycin-resistant mutants could be selected from the rapamycin-sensitive Jurkat T cell line.

Both rapamycin-sensitive and rapamycin-resistant cell lines showed comparable FKBP-12 activity, assessed by immunoblotting and by quantitative rapamycin absorption studies. Investigation of the kinetics of rapamycin action demonstrated that inhibition of proliferation was preceded by inhibition of cellular protein synthesis. Rapamycin did not affect the progression into S phase of mimosine-arrested Jurkat cells.

I 544 CYCLIC AMP INHIBITS PHOSPHATIDYLINOSITOL-COUPLED AND UNCOUPLED MITOGENIC SIGNALS IN T LYMPHOCYTES. EVIDENCE THAT cAMP ALTERS PKC-INDUCED TRANSCTIPTION REGULATION OF MEMBERS OF THE *jun* AND *fos* FAMILY OF GENES

Ami Tamir and Noah Isakov, Department of Microbiology and Immunology, Ben Gurion University of the Negev, Beer Sheva, Israel

T lymphocyte stimulation via the antigen receptor results in activation of phospholipase Cγ1 that catalyses the hydrolysis of phosphatidylinositol (PI). The hydrolysis generates inositol phosphate and diacylglycerol, which in turn increase intracellular Ca^{2+} concentration and activate protein kinase C, respectively. Agonists operating via the adenylate cyclase pathway or cell permeable cAMP analoges inhibit T cell activation by interfering with the PI turnover. We have shown that dbcAMP inhibits PI-independent mitogenic signals in murine allo-reactive T cells stimulated with IL2 or TPA+ionomycin. dbcAMP inhibited the TPA+ionomycin induced transcription of IL2 and IL2-receptor genes suggesting interference with biochemical events downstream to PI hydrolysis and upstream to transcription of 'early' genes. Since many of the 'early' genes operating in T cell mitogenesis posses TPA responsive element (TRE) in their promoter region we tested the effect of cAMP on the TRE binding protein, AP-1. dbcAMP increased binding activity of nuclear proteins consisting of Jun:Fos heterodimers to a TRE-containing oligonucleotide, but altered the composition of Jun proteins in the AP-1. Furthermore, the TPA+ionomycin-induced transcription program of members of the *jun* and *fos* family of genes was altered by dbcAMP suggesting that its inhibition of T cell proliferation is a consequence of intervention in transcriptional regulation by TRE binding proteins.

I 545 EVI-1 RAISES AP-1 ACTIVITY IN THE SECOND ZINC FINGER DOMAIN-DEPENDENT MANNER,

Tomoyuki Tanaka, Junji Nishida, Kinuko Mitani, Yoshio Yazaki, Hisamaru Hirai, Department of Molecular Biology, Jichi Medical School, Tochigi; The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo, Japan

Evi-1 was originally identified as the gene associated with a common viral integration site in murine leukemias. The expression of Evi-1 is induced also in some human acute myeloid leukemias. We analyzed the relationship between Evi-1 and cellular proliferation signals. When Evi-1 was expressed, trans-activation of TPA-responsive element and increased c-jun expression were observed in NIH3T3 and P19 cells. In addition, P19 cells transfected with Evi-1 showed some differentiation phenotypes. These phenotypes resembled those observed in activation of AP-1 in P19. These studies indicate that Evi-1 raises AP-1 activity. Besides, the upstream sequence of c-fos was trans-activated in the presence of Evi-1. This is probably one of mechanisms for AP-1 activation by Evi-1. Evi-1 has 7 zinc fingers at the N-terminal (first zinc finger domain) and 3 zinc fingers near the C-terminal (second zinc finger domain). We constructed deletion mutants of Evi-1 and investigated functions of zinc fingers. It was shown that the second zinc finger domain is essential for AP-1 activation and trans-activation of c-fos promoter. Further functional analyses of Evi-1 domains are now under investigation.

I 546 SIGNALS THAT CONTROL APOPTOSIS RESULT IN ALTERED REGULATION OF THE MAP KINASE CASCADE, Mary A. Valentine, Rony Seger, Jean Campbell, Amy M. Jensen, and Edwin G. Krebs, Departments of Microbiology and Pharmacology, University of Washington, Seattle, WA 98195

Ligation of the antigen receptor on human B cells (surface IgM) can result in a variety of cellular responses including proliferation, differentiation, or cell death by apoptosis. We have examined the amplification systems used by the B cell line Ramos to determine which protein serine/threonine pathways are influenced when this cell is signaled to become apoptotic and how this may differ from the events associated with a proliferative response. Normal resting B cells respond to ligation of the antigen receptor with activation of several independent kinases, including activation of a family of mitogen activated protein kinases (aka MAP kinases or ERKs) that function as part of an activation cascade. We and others have identified the sequence of events of this cascade to involve the MAP kinase kinase (MAPKK), which phosphorylates and activates MAP kinase, that can then activate the $p90^{rsk}$, an enzyme that can phosphorylate the S6 protein of the 40S ribosomal subunit. Using conditions of antigen receptor ligation on Ramos cells that result in programmed cell death (PCD), we found an extremely vigorous activation of the MAPKK with no subsequent increase of the low basal level of MAPK or activation of the $p90^{rsk}$. Interestingly, ligation of surface IgM abrogated basal MAPK activity within 30 min, suggesting activation of an inducible inhibitor in these cells. We have previously reported that PCD can be avoided by ligation of the surface protein CD40 on Ramos cells. Therefore, it was of interest to determine if anti-CD40 would restore the integrity of the cascade concurrent with its ability to override apoptosis. We found that ligation of CD40 could strongly activate the MAPK while having no effect on the MAPKK. These results suggest the presence of a MAPK activator unique from the MAPKK and lend correlative evidence for the importance of an intact MAPK cascade to avoid PCD.

I 547 ASSOCIATION OF THE 60 kDa TNF RECEPTOR WITH A LIGAND STIMULATED SERINE KINASE ACTIVITY. Todd L. VanArsdale, and Carl F. Ware. Division of Biomedical Sciences, University of California, Riverside, CA 92521.

While signalling pathways activated in response to TNF are beginning to be elucidated, signalling activities coupled to either of the two defined TNF receptors, $TNFR_{60}$ and $TNFR_{80}$, have not been clearly demonstrated. We have analyzed $TNFR_{60}$ immune complexes prepared from $^{32}PO_4$ labeled cells or labeled within in vitro immune complex kinase reactions to determine whether protein kinases play a role in receptor associated signalling activities. Results show that $TNFR_{60}$ immune complexes prepared from $^{32}PO_4$ labeled U-937 or HL-60 cells with affinity purified IgG contain TNF dependent co-precipitating phosphoproteins of 126 and 95 kDa, suggesting the phosphorylation of proteins associated with $TNFR_{60}$ following the ligand binding event. To further analyze receptor associated proteins $TNFR_{60}$ immune complexes were subjected to in vitro kinase reactions which have demonstrated the ligand dependent association of a protein serine kinase activity with $TNFR_{60}$. This kinase activity results in the serine phosphorylation of proteins contained within the immune complex migrating at 126, 95, 80 and 62 kDa on SDS-PAGE. When ligand-receptor complexes are isolated with anti-TNF sera a similar pattern of phosphoproteins are detected. Kinase activity is stimulated with one minute of cellular TNF exposure and reaches near maximal levels with TNF concentrations of 10pM. The demonstration of protein kinase activity stimulated in a time and dose dependent manner in respect to cellular TNF exposure and co-purifying with $TNFR_{60}$ suggests a protein serine kinase plays a role in the initial signalling events mediated through $TNFR_{60}$. Supported by TRDRP RT0261 and ACS grant IM68167.

I 548 MATURATION OF STIMULUS-TRANSCRIPTION COUPLING PATHWAYS ASSESSED IN FOS-LACZ TRANSGENIC MICE, M. Vendrell, L.Robertson, T. Curran and J.I. Morgan. Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110.

Extracellular stimuli elicit the rapid, transient, transcriptional activation of a class of genes known as cellular immediate-early (cIE) genes. Many of these genes encode nuclear proteins, that are known, or putative, transcription factors such as Fos and Jun, the products of the proto-oncogenes c-*fos* and c-*jun*. Fos-Jun heterodimers regulate transcription of target genes by binding to AP1 sites. In this sense the cIE gene products behave as if they were nuclear third messengers, coupling short term extracellular stimuli to longer term alterations in cellular phenotype by changing target gene expression. To pursue the issue of cIE gene expression *in vivo* we have developed a fos-lacZ transgenic mouse that recapitulates c-*fos* expression. Our lab has already shown that constitutive expression of c-*fos* in certain regions of the nervous system during development seemed to be associated with the acquisition of new physiological responses. This led us to determine whether c-*fos* expression could be induced at early postnatal ages. We have administered the excitotoxin, kainate, to developing transgenic mice and studied Fos-lacZ expression in the hippocampus. At postnatal day 2 (P2) the transgene is non-inducible despite the fact that behavioral effects are evident. At P5, fos-lacZ is induced specifically in CA3 pyramidal neurons. On subsequent days of development there is a sequential induction in the dentate gyrus by P10 and finally the CA1 neuronal layer by P15. This developmental pattern closely reflects the cellular and physiological maturation of these structures. To determine whether this involves maturation of particular elements of the signal transduction cascade, rather than physical growth of neurons, we have assessed inducibility of fos-lacZ in mice that have specific point mutations in the promoter of the transgene. These data imply that while the majority of the changes reflect the appearance of glutamate receptors some aspects involve maturation of intracellular signalling pathways.

I 549 TAXOL-INDUCED APOPTOSIS: PROGRESSION OF CELL CYCLE REGULATION IN THE ABSENCE OF MITOSIS.

Alan F. Wahl, Karen Donaldson, Gay Lynn Goolsby and Peter A.Kiener, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121

Tumor cells exposed to the antimicrotubule agent taxol provide an excellent model for cellular processes leading to cell death by apoptosis. Transformation of normal fibroblasts by SV40 virus increases cell sensitivity to taxol by 140-fold, thus we have examined events following taxol exposure in synchronized tumor cells that are participant in apoptosis. Within synchronized populations, cells in transition from G_0 to G_1 and cells at G_2/M phase are most sensitive to taxol and a stable taxol-tubulin complex exists in transition and mitosis. Mitotic arrest is not be requisite for cell death, as G_2/M phase cells or G_1/S-phase cells treated with taxol synchronously initiate apoptotic DNA fragmentation within 12 h of treatment. Treatment at G_1/S or G_2/M clearly blocks mitosis and subsequent DNA synthesis, however, a progression of cell cycle regulated events including protein phosphorylation and cyclin dependent $p34^{cdc2}$ kinase activation continue as in untreated cells. This progression appears requisite as apoptosis of cells blocked at mitosis is reduced by cyclohexamide and by both inhibitors of protein kinase and phosphatase, suggesting both protein synthesis and modification are required for cell death. Onset of DNA fragmentation is coincident with decreased intracellular pH. Taken together, these results suggest the taxol lesion is not sufficiently recognized in tumor cells to stop cell cycle progression, and so dissociation of temporally linked cell cycle events may trigger apoptosis.

I 550 ANALYSIS OF A HUMAN PROTEIN KINASE THAT PHOSPHORYLATES TYR15 IN CYCLIN DEPENDENT KINASES

Nobumoto Watanabe, Martin Broome and Tony Hunter

Molecular Biology and Virology Laboratory, The Salk Institute for Biological Studies, P.O. Box 85800, San Diego, California 92186

In vertebrates, cyclin dependent kinases (CDKs), the catalytic subunits of cyclin/CDK complexes, are regulated negatively by phosphorylation on T14,Y15 and positively by phosphorylation on T161 (in cdc2) or its equivalent residue. To study the CDK Y15-kinase, we raised an antiserum against the C-terminal peptide of the human Wee1-like kinase which was cloned by Okayama's group through its ability to complement a wee1-deficient fission yeast mutant. The antiserum detected a 93/95 kD doublet in HeLa cells, which is significantly larger than the size (49 kD) of the protein expressed from the reported sequence. We speculated that the reported cDNA is incomplete, and upon re-screening a human cDNA library we obtained a longer cDNA with an open reading frame extending 214 amino acids upstream with an in-frame stop codon in the 5' non-translated region. We confirmed that this cDNA contains the complete ORF by *in vitro* transcription and translation analysis and transient expression in COS cells. Although the protein sequence has weak homology with Wee1 in its C-terminal kinase domain, it has no homology with Wee1 in its N-terminal domain. However we found weak but significant homology with fission yeast Mik1 (Mitosis Inhibitory Kinase which has similar role to Wee1) over its entire length including the noncatalytic N-terminal domain. Therefore, we suggest that this is not human homologue of Wee1 but of Mik1. The human Mik1 immunoprecipitated from HeLa cells phosphorylates cyclin-complexed CDKs exclusively on Y15. In addition we found that cyclin-CDK phosphorylates Mik1 in its N-terminal region and regulates its activity. Analysis of cell cycle dependent changes in Mik1 activity is now in progress.

I 551 CLONING OF NOVEL ONCOGENIC PROTEINS BY RETROVIRAL VECTOR-MEDIATED TRANSFER AND EXPRESSION OF cDNA LIBRARIES. Ian Whitehead, Heather Kirk and Robert Kay, Department of Medical Genetics, University of British Columbia and Terry Fox Laboratory, British Columbia Cancer Agency, 600 W. 10th Avenue, Vancouver, BC, Canada, V5Z 4E6.

Procedures have been developed that enable large cDNA libraries to be converted into retroviruses and then transferred to mammalian cells by infection. This approach is being used to screen for cDNAs that transform recipient fibroblast and epithelial cell lines. Doping experiments have demonstrated that oncogenic cDNAs present at very low abundances in transferred libraries can be readily detected by the induction of transformed foci of recipient cells, and that the cDNAs within the proviruses in the transformed cell clones can be rapidly and specifically isolated and recloned by PCR. Transfer of retroviral cDNA libraries of several hundred thousand clones resulted in the induction of numerous transformed foci with various morphologies. cDNAs recovered from transformed cells proved to have transforming activity when individually transferred into secondary recipient cells. Some of these cDNAs encode known oncogenes, while others encode full-length or partial proteins that were either previously unidentified or had not been known to have oncogenic potential. The mechanisms by which these proteins transform cells are now under investigation.

The use of retroviral vectors allows the transfer of cDNA libraries to many different cell types, and therefore this expression cloning system should be uniquely effective for cloning molecules that impose selectable alterations in diverse cell phenotypes.

I 552 EPIDERMAL GROWTH FACTOR DOWN-REGULATES THE EXPRESSION OF GLYCINE tRNA SYNTHETASE IN CULTURED HUMAN MESANGIAL CELLS. James Williams, Tee Fern Khong, Sarah Osvath, Martin Pearse and David Power. Department of Clinical Immunology, St Vincent's Hospital, 41 Victoria Parade, Fitzroy 3065, Victoria, Australia.

Proliferative glomerulonephritis is characterised by mesangial cell proliferation and altered extracellular matrix (ECM) production. Several cytokines have been reported to induce proliferation of cultured mesangial cells, including epidermal growth factor (EGF). PCR-based subtractive hybridisation was used to identify genes whose transcription is affected by EGF in cultured human mesangial cells. One of two cDNA species found to be down-regulated by EGF (clone B26) encodes a 2.7kbp mRNA species whose expression is reduced 2-3 fold following stimulation of the cells with EGF. A search of the nucleotide databases showed that clone B26 has 60% identity over 917bp to the silk worm glycine-tRNA synthetase gene. The predictted translation product of clone b26 shares >80% homology at the amino acid level with the silk worm glycine-tRNA synthetase. Clone B26, therefore, appears to encode part of the human glycine-tRNA synthetase gene; a gene not previously sequenced in vertebrates. tRNA synthetases are multi-functional proteins whose major function is to load amino acids onto tRNA. They are specific for each amino acid. Glycine-tRNA synthetase was originally identified in the silk worm because it is up-regulated in the silk gland, so increasing the availability of gly-tRNA for the synthesis of silk, a glycine rich protein. There is no comparable evidence for regulation of this gene in humans. Moreover, preliminary reports from other investigators have shown that EGF decreases collagen synthesis by cultured mesangial cells. Since glycine represents every third amino acid in the repeating unit of the collagen chains, down-regulation of glycine tRNA synthetase mRNA by EGF may regulate collagen synthesis through decreased availability of gly-tRNA molecules. Alternatively, reduced glycine tRNA synthetase may be a response to decreased gly-tRNA utilization. Future studies will investigate the signal transduction pathway involved in cytokine regulation of human gly-tRNA synthetase expression and, hence, determine which of these possibilitis is correct.

I 553 ROLE OF KIT-LIGAND IN PROLIFERATION, AND SUPPRESSION OF APOPTOSIS INDUCED BY GROWTH FACTOR- DEPRIVATION AND γ-IRRADIATION, IN MAST CELLS, Nelson S. Yee, Inbok Paek* and Peter Besmer, Molecular Biology Program, Sloan-Kettering Institute and Cornell University Graduate School of Med. Sci., *Dept. of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

The receptor tyrosine kinase c-*kit* and its cognate ligand KL/steel factor are encoded at the *white spotting (W)* and *steel (Sl)* loci of the mouse, respectively. Mutations at both *W* and *Sl* loci cause deficiencies in hematopoiesis including the stem cell hierarchy, erythropoiesis and mast cells, as well as gametogenesis and melanogenesis. In addition mutant mice display an increased sensitivity to lethal doses of irradiation. The role of KL/c-*kit* in cell proliferation and survival under conditions of growth factor-deprivation and γ-irradiation was studied by using bone marrow-derived mast cells (BMMC) as a model. Either in the presence or absence of serum, KL stimulated mast cell proliferation in a dose-dependent manner. At a relatively lower concentration, KL prevented apoptosis induced by both growth factor-deprivation and γ-irradiation and suppressed the internucleosomal DNA fragmentation characteristic of apoptosis. Analysis of BMMC heterozygous for various W alleles indicated that the level of c-*kit* receptors was dose-limiting in promoting the proliferative response to KL. For cells to progress through the cell cycle presence of KL was required only during the initial 8-10 h of the 12-14 h duration of G_1 phase for entry into S phase. However continuous KL presence was required for complete suppression of apoptosis due to deprivation of KL. The radioprotective effect of KL was independent of the phase of the cell cycle in which the cells were irradiated and addition of KL to γ-irradiated cells could be delayed for up to 1 h before there was a significant increase in radiation-induced apoptosis. It is proposed that the increased sensitivity to lethal irradiation of *W* and *Sl* mutant mice results from paucity of the apoptosis suppressing effects of KL.

I 554 IDENTIFICATION OF A NOVEL PROTEIN INTERACTING WITH BCL-2, Jiping Zha, Elizabeth Yang, and Stanley J. Korsmeyer, Howard Hughes Medical Institute, Washington University School of Medicine, St. Louis, MO 63110

Bcl-2 protein represses a number of apoptotic death pathways. To understand the molecular mechanism of its function, we investigated whether Bcl-2 protein interacts with other proteins. A novel gene, beyond Bax, was identified by interactive cloning by both a 2 hybrid yeast system and by screening an expression library with radiolabeled Bcl-2 protein. This protein interacted with labeled GST-Bcl-2 fusion protein, but not labeled GST protein by Far Western analysis. Deletion analysis defined the interacting motif to a 11-amino acid region which shares homology with Bcl-2 homology domain I (BH1). Site-directed mutagenesis within this region is being used to determine the contribution of individual residues to its interaction with Bcl-2. A similar approach will be applied to localize the interaction domain within the Bcl-2 protein. The biological effects of this wild type protein and its mutants are under analysis.

I 555 SEPARATION OF FRAGMENTS OF EQUAL LENGTH BY SOUTHERN BLOT HYBRIDIZATION AND SUBSEQUENT PCR AMPLIFICATION OF THE SIGNAL, Zickert P, Blegen H, Latham C and Zetterberg A., Department of Pathology, Divition of Tumor Pathology, The Karolinska Hospital, S-10401Stockholm, SWEDEN.

We describe a fast and simple protocol, called Southern blot PCR (SB-PCR), for separation of complex PCR products of equal length obtained by reverse transcriptase-PCR (RT-PCR) using degenerate primers for multigene families. By using degenerate primers for multigene families, such as protein tyrosin kinases (PTK), in RT-PCR, a certain number of different DNA molecules are amplified depending on the phenotype. The resultant mixture of amplified PTK-gene fragments are of equal length and cannot be fractionated on an agarose or polyacrylamide gel. The downstream primers are biotinylated in order to immobilize the complex PCR products on streptavidin coated magnetic beads. This enables removal of the complementary nonbiotinylated strand and single stranded radioactive probes are subsequently made by a extension reaction using the upstream primer. Several bands, depending on the fenotype, are seen using this complex radioactive probe in a Southern blot hybridization. After Southern blot hybridization, the signal/single-stranded-probe containing area are identified and cut out from the filter. The excised filters are used as targets for DNA reamplification. The reamplified product is then sequenced directly. This technique is useful in experiments where the gene expression pattern of gene families are analyzed such as tumour versus normal tissue or cell cycle positions. Differentially expressed genes are thus seen directly after Southern blot hybridization and the signals could be excised from the filter for reamplification and sequence determination. In this poster we have used three subfamilies of the protein tyrosine kinase family to demonstrate this method.

Late Abstracts

THE FUNCTION OF CD44 IN T CELL ACTIVATION

Robert Arch and Margot Zöller, German Cancer Research Center, Department of Immune Regulation and Tumor Defense, Heidelberg, Germany

CD44, originally described as a lymphocyte homing receptor, in fact is a family of glycoproteins which vary by glycosylation and protein structure, mainly due to alternative splicing. Up to 10 different variant exons can be inserted in the so called hemopoetic or standard form of CD44 (CD44s). While the majority of lymphocytes expresses CD44s, expression of splice variants of CD44 could be observed only during maturation and activation of lymphocytes. Cloning and sequencing of lymphocytes subpopulations revealed that antigen presenting cells, B cells, and T cells express different splice variants of CD44. Upregulation during lymphocyte activation is strictly regulated. Upon mitogenic stimulation *in vitro*, CD44 mRNA is detected after 50-60 hours and protein expression, as revealed by staining with a monoclonal antibody recognizing an epitope on exon v6 is noted after 60 hours. There is evidence that CD44s does not merely function as adhesion molecule, instead is directly involved in the activation process, since activation of lymphocytes can be inhibited by anti-CD44v antibodies. Anti-CD44v mediated inhibition of lymphocyte activation functions solely when the antibody is present during the starting period of the activation and only in the presence of presenting cells during antigen-specific activation, while mitogenic activation is not influenced by anti-CD44v treatment. According to the time course of expression and the features of antibody interference, it appears likely that different splice variants of CD44 exert different different functions on antigen presenting and helper/effector lymphocytes. We are currently proving our working hypothesis that variants of CD44 as expressed on antigen presenting cells initiate growth factor / cytokine production, where function may be optimized upon crosslinking between variant CD44 molecules on antigen presenting cells and lymphcytes.

A SITE ON TRANSDUCIN α-SUBUNIT OF INTERACTION WITH THE POLYCATIONIC REGION OF cGMP-PHOSPHODIESTERASE γ-SUBUNIT. Nikolai O. Artemyev#, John S. Mills#, Kelly R. Thornburg@, Daniel R. Knapp@, Kevin L. Schey@, and Heidi E. Hamm#, #Department of Physiology and Biophysics, University of Illinois College of Medicine, Chicago, Illinois 60680 and the @Department of Cell and Molecular Pharmacology, Medical University of South Carolina, Charleston, South Carolina 29425-2251.

Activation of cGMP-phosphodiesterase (PDE) by the rod G-protein transducin is a key event in visual signal transduction in vertebrate photoreceptor cells. Interaction between the GTP-bound form of the α-subunit of transducin (α_t*) and the PDE inhibitory γ-subunit (Pγ) is a major component of PDE activation. We are interested in studying this interaction as a model for G protein-effector function. The central polycationic region of Pγ, Pγ-24-45, has been implicated as one of the sites involved in α_t*-Pγ interaction. Here we determine the site on α_t* that interacts with Pγ-24-45 using a photo cross-linking approach. The synthetic peptides Cys(ACM)Tyr-Pγ-24-45-Cys and Cys-Pγ-24-45 were labeled with 4-(N-maleimido) benzophenone at C- and N-terminus respectively and then cross-linked to α_t. When the photoprobe was attached to the C-terminus of the peptide, a specific high yield cross-linked product (80%) was formed between the peptide and α_tGTPγS. A lower yield of cross-linking (35%) was seen between the peptide and α_tGDP. The site of cross-linking between Cys(ACM)Tyr-Pγ-24-45-Cys and α_tGTPγS was localized to within α_t-306-310 using a variety of chemical and proteolytic cleavages of the cross-linked product and analysis of the fragments with SDS-PAGE and matrix-assisted laser desorption ionization mass spectrometry. We believe that such combination of cross-linking and mass spectrometric analysis of cross-linked product fragments represents a new promising approach for studying protein-protein interactions.

SUBCELLULAR LOCALIZATION OF PI3-KINASE: EVIDENCE FOR ACTIN ASSOCIATION AND PRESENCE IN NUCLEI AND MITOCHONDRIA. Paul van Bergen en Henegouwen[1], Johannes Boonstra[1], Arie J. Verkleij[1], *Hughes Chap*[2] and Bernard Payrastre[2]. [1]Dept. Molecular Cell Biology, University of Utrecht, The Netherlands and [2]INSERM Unit 326, Hôpital Purpan, Toulouse, France.

Phosphatidylinositol 3-kinase (PI3-kinase) is a heterodimer consisting of the adaptor protein p85 and the catalytic protein p110. In mammalian cells, two isoforms of p85 are present, p85α and p85β. Here we present our investigations on the subcellular localization of PI3-kinase in Swiss 3T3 cells. PI3-kinase was localized using a combined approach of immunocytochemical and cell fractionation techniques. For immunolocalization an antibody directed against p85α was used. The different cellular fractions were analyzed by western blotting and by enzymatic analysis for PI3-kinase activity. We found that p85α colocalizes with stress fibers. After isolation of actin filaments we observed that PI3-kinase can bind to these filaments *in vitro*. Moreover, PI3-kinase was found in the nucleus in association with the internal matrix. Finally, PI3-kinase was observed in the mitochondria of Swiss 3T3 cells.

INSERTION MUTAGENESIS STUDIES OF THE m3 MUSCARINIC ACETYLCHOLINE RECEPTOR PROVIDE EVIDENCE FOR AN α-HELICAL STRUCTURE OF A G PROTEIN ACTIVATOR REGION, Klaus Blüml and Jürgen Wess, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD 20892

Several lines of evidence suggest that the N-terminal portion of the third cytoplasmic loop (i3) of muscarinic and other G protein-coupled receptors is of pivotal importance for G protein recognition and activation. Whereas secondary structure algorithms predict that this region may form an amphipathic α-helical extension of the fifth transmembrane domain, recent studies using small peptides derived from this receptor segment have cast considerable doubt on this notion (Voss et al., *J. Biol. Chem.* 268, 4637, 1993). Assuming that the N-terminal portion of the i3 loop in fact adopts an α-helical secondary structure, we speculated that rotation of this helical domain (e.g., by insertion mutagenesis) should have pronounced effects on receptor/G protein coupling. To test this hypothesis, we constructed a series of mutant rat m3 muscarinic receptors [(m3(+1A) to m3(+5A)], in which 1, 2, 3, 4, or 5 additional alanine residues were inserted at the beginning of the i3 domain (following Arg252). The ability of the various mutant receptors to mediate agonist-induced stimulation of phosphatidyl inositol (PI) turnover was studied in transiently transfected COS-7 cells. Whereas m3(+1A) was able to stimulate PI hydrolysis to the same maximum extent as the wild type m3 receptor, m3(+2A) proved to be functionally completely inactive. Interestingly, m3(+3A) and m3(+4A) regained considerable functional activity (E_{max} ca. 70% of wild type m3). However, m3(+5A) again lost the ability to induce an appreciable PI response. Helical wheel models show that our experimental data are fully consistent with the notion that the N-terminal portion of the i3 loop adopts an α-helical conformation and that the hydrophobic side of this helix represents the G protein recognition surface. These findings should pave the way towards a better understanding of the molecular details of receptor/G protein interactions.

A new function for a phosphotyrosine phosphatase: linking GRB2/Sos to a receptor tyrosine kinase.

Wei Li, Riko Nishimura, Adam Kashishian+, Andreas Batzer, William J. H. Kim, Jonathan A. Cooper+ and Joseph Schlessinger Department of Pharmacology, New York University Medical Center, 550 First Avenue, New York, NY, 10016, and +Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104.

Autophosphorylated growth factor receptors provide binding sites for the SH2 domains of intracellular signaling molecules. In response to epidermal growth factor (EGF), the activated EGF receptor binds to a complex containing the signaling protein GRB2 and the Ras guanine nucleotide releasing factor Sos, leading to activation of the Ras signaling pathway. We have investigated whether the platelet-derived growth factor (PDGF) receptor binds GRB2/Sos. In contrast with the EGF receptor, the GRB2 does not bind to the PDGF receptor directly. Instead, PDGF stimulation induces the formation of a complex containing GRB2, 70- 80- and 110-kDa tyrosine-phosphorylated proteins and the PDGF receptor. Moreover, GRB2 binds directly to the 70 kDa protein but not to the PDGF receptor. Using a panel of PDGF β-receptor mutants with altered tyrosine phosphorylation sites, we identified tyr-1009 in the PDGF receptor as required for GRB2 binding. Binding is inhibited by a phosphopeptide containing a YXNX motif, a sequence that is not found in the PDGF receptor. The protein tyrosine phosphatase Syp/PTP1D/SHPTP2/PTP2C is approximately 70 kDa, binds to the PDGF receptor via tyr-1009, and contains several YXNX sequences. We found that the 70 kDa protein that binds to the PDGF receptor and to GRB2 co-migrates with Syp and is recognized by anti-Syp antibodies. Furthermore, both GRB2 and Sos co-immunoprecipitate with Syp from lysates of PDGF-stimulated cells, and GRB2 binds directly to tyrosine phosphorylated Syp in vitro. These results indicate that GRB2 interacts with different growth factor receptors by different mechanisms and the cytoplasmic phosphotyrosine phosphatase Syp acts as an adapter between the PDGF receptor and the GRB2/Sos complex.

DETERMINATION OF KINETICS OF GUANINE NUCLEOTIDE INTERACTION WITH G_t USING STOPPED-FLOW FLUORESCENCE WITH MANT GTPγS AND LUCIFER YELLOW PDEγ.

John S. Mills and Heidi Hamm. Department of Physiology and Biophysics, University of Illinois at Chicago, Chicago Illinois, 60680.

The interaction of guanine nucleotides with G_t has been investigated using stopped flow fluorescence techniques. PDEγ subunit was labeled at its single cysteine with lucifer yellow vinyl sulfone (LYPγ). GTPγS was labeled with methylisotic anhydride to produce 2' (3') methyanthranoyl GTPγS. Both were purified to >95 % purity using HPLC. LYPγ interacts specifically with the activated form of G_t, producing a 2.5 fold increase in fluorescence (Artemyev et al., J. Biol. Chem. 267, 25067 ,1992) and was used to observe the time dependence of G_t activation by GTP, GTPγS, GMPPNP, and GMPPCP. All were capable of activating G_t as measured by its ability to enhance the fluorescence of LYPγ, but the rates of activation differed markedly with activation rates by GTPγS>GTP>GMPPNP>GMPPCP. The maximal observed rate with GTPγS was ~30/sec with maximal rate observed at 2 uM and half-maximal rate at 0.2 uM. A similar maximal rate was observed with GTP, but 20 uM GTP was necessary to produce half-maximal rate. 20 uM GMPPNP activated G_t 20 times more slowly than did 0.2 uM GTPγS implying an efficacy 3 orders of magnitude less than GTPγS. GMPPCP activated G_t 4 orders of magnitude more slowly than did GTPγS. When hydrolyzable GTP was used, the time dependence of G_t inactivation was also determined. Inactivation of G_t occurred at 0.15/sec, 0.07/ sec and 0.03/sec at 25 uM, 10 uM, and 4 uM bleached rhodopsin, respectively. Guanine nucleotide binding to G_t was also observed using methylanthranoyl labeled GTPγS (MANTGTPγS). At 20 uM, a fast phase of binding occurred with a rate of ~30/sec followed by several slower rates, implying that the binding and activation occur in several steps. Simultaneous monitoring in the presence of LYPγ showed that G_t binding and activation of LYPγ occurred subsequent to MANTGTPγS binding rather than concomitantly. The time trace using LYPγ in the presence of GTPγS also exhibited at least three time phases, again implying a multistep reaction. Thus both fluorescent probes provide evidence of a rate limiting step in G_t activation of about 30/sec which probably represents the maximal rate at which G_t can be activated. This rate is consistent with observed onset times of ~50 msec observed by measuring of membrane potentials.

THE HEPATITIS B VIRUS TRANSACTIVATOR pX ACTIVATES C-JUN TRANSCRIPTION FACTOR THROUGH A RAS- AND RAF-DEPENDENT INTRACELLULAR SIGNALLING PATHWAY.

*G. Natoli^, P. Chirillo^, M.L. Avantaggiati^, A. Costanzo^, A. Ianni^, E. De Marzio^, P.L. Puri^, M. Artini^, C. Balsano^, M. Levrero^**.
^Istituto di I Clinica Medica e Fondazione A. Cesalpino, Universita' di Roma La Sapienza. * Istituto di Medicina Interna, Univ. di Cagliari.

The Hepatitis B Virus X protein is a promiscuous transactivator of RNA polymerase II as well as RNA polymerase III promoters, whose mechanisms of action are poorly understood. Previous reports suggested the possibility of a direct interaction between pX, which is devoid of DNA binding activity, and components of the cellular transcription machinery (such as CREB/ATF2), and it was shown that pX might indeed possess an acidic domain that makes it able to activate transcription when fused to the DNA binding domain of the liver restricted transcription factor C/EBP. Recently, to better explain the promiscuity of pX activity on transcription, alternative models, based on the activation of cellular kinases involved in growth regulation and control, have been proposed. We investigated the mechanisms of c-Jun activation by pX and in particular the role of cellular proteins involved in signal transduction (namely Ha-Ras and Raf-1). In both HeLa and undifferentiated F9 cells pX was able to increase the activity of exogenous transfected wild-type c-Jun, but not of c-Jun mutants bearing mutations in the amino-terminal serine residues. We show, by use of HaRas and Raf-1 dominant negative mutants that both HaRas and Raf1 are required for pX-induced activation of c-jun transcriptional activity. In addition we show that pX is able to cooperate with Raf-1 in c-Jun activation. Our results are consistent with the hypothesis that at least one site of action of pX is peripheral and is located upstream of the Ras genes products.

CHARACTERISATION OF PHOSPHORYLATION EVENTS GOVERNING THE DNA-BINDING ACTIVITY OF ETS-1

Bertrand R. Rabault and Jacques Ghysdael, CNRS URA 1443 , Institut Curie, Centre Universitaire, 91405 ORSAY Cedex.

Ets-1, the product of the c-ets-1 proto-oncogene, is the prototype of a family of transcriptional regulators the activity of which depends on the binding to specific DNA sequences (EBS, Ets binding sites). Ets-1 is preferentially-but not exclusively- expressed in cells of the lymphoid lineage. Our previous studies have shown that engagement of the antigen receptor of both T and B cells resulted in the rapid and transient hyperphosphorylation of Ets-1 on serine residues and that these events were strictly dependent upon the increased concentration of Ca_i^{++} which occurs during activation of these cells (Pognonec et al., (1988) EMBO J.,7, 977-983.).

Using a combination of two dimensional tryptic phosphopeptides analyses and site-directed mutagenesis, we report the identification of four serine residues in Ets-1 which are hyperphosphorylated in transfected COS cells following treatment of these cells with ionomycin. These serine residues were found to be clustered in a region of Ets-1 adjacent to its DNA binding domain (Ets domain). Unlike the wild type protein which looses its ability to bind to EBS after treatment of the cells with ionomycin, the mutant protein in which the four identified serines are mutated into alanine was found to be unaffected in its DNA binding capacity when analysed in similar conditions. These results indicate therefore that the calcium induced phosphorylation of Ets-1 inhibits its DNA-binding activity.

RECONSTITUTION OF THE B CELL ANTIGEN RECEPTOR SIGNALING COMPLEX IN COS CELLS. Sandra J. Saouaf, Sandeep Mahajan, Stephanie A. Kut, R. Bruce Rowley, Joseph B. Bolen, and Anne L. Burkhardt. Signal Transduction Laboratory, Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute. Princeton, NJ 08543

Crosslinking of the B cell antigen receptor on mature cells results in activation and proliferation. Changes within the cells can be detected within seconds of antigen receptor engagement. Hydrolysis of phosphatidyl inositol takes place as well as mobilization of intracellular calcium. Phosphorylation of proteins on tyrosine, including the antigen receptor signaling components Igα and Igβ, is one of the earliest detectable events and indicates the activation of tyrosine kinases. Many non-receptor tyrosine kinases are expressed in B cells including blk, lyn, fyn, hck, and syk. We have demonstrated that several src-related protein tyrosine kinases become activated upon receptor crosslinking. In addition, the tyrosine kinases blk, lyn and fyn can be co-immunoprecipitated with the immunoglobulin (Ig) receptor.

In order to determine both the functional and physical association of the Ig receptor signaling components with the individual non-receptor tyrosine kinases, we have expressed in Cos cells the kinases and a chimeric protein containing the extracellular domain of the platelet derived growth factor receptor with the transmembrane and intracellular domain of Igα or Igβ. The chimeric Igα and Igβ proteins are expressed on the surface of transfected Cos cells and can be detected by Western blotting of cell lysates. The transfected tyrosine kinases are enzymatically active as assessed by their ability to autophosphorylate in an *in vitro* kinase assay as well as by their ability to phosphorylate an exogenously added substrate (gst-Igβ). Co-transfections of kinases and the chimeric receptor proteins are in progress to assess the kinase-substrate interactions. In addition, mutations in both the Igα and Igβ chimeras as well as in the tyrosine kinases are being analysed.

Using this approach we will be able to determine some of the possible Ig receptor-tyrosine kinase interactions which take place within B cells.

Characterization of a novel src-family tyrosine kinase expressed in the intestine. Valeri Vasioukhin, Michael S. Serfas, Elena Siyanova, Marina Polonskaia, and Angela L. Tyner, Dept. of Genetics, University of Illinois College of Medicine, Chicago, Illinois.

To identify tyrosine kinases that may be involved in the regulation of the regeneration of the mammalian intestinal epithelium, we amplified portions of the catalytic domains of protein kinases expressed in intestinal crypt cells, using the polymerase chain reaction technique with primers directed against two invariant amino acid sequence motifs found in all kinases. These fragments were cloned and a library of kinase catalytic domains was generated. Sequence analysis of unique clones resulted in the identification of the catalytic domains of several characterized tyrosine kinases, including lyn, hck, c-fgr, tec, JAK2 , itk, and the putative receptor kinase ryk. Expression of these kinases has not previously been reported in the intestine. Clones encoding two novel catalytic domain sequences were also identified. One of these which we have named sik, for **s**rc-family **i**ntestinal **k**inase, shares highest homology with members of the src subfamily of tyrosine kinases, although it contains the sequence Asp-Leu-Ala-Ala-Arg-Asn in its catalytic domain, not Asp-Leu-Arg-Ala-Ala-Asn, like other vertebrate members of the src-family. When compared with sequences in Genbank, sik shares greatest homology with a family of src-related kinase genes isolated from the fresh water sponge *Spongilla lacustris*, the simplest organism to contain tyrosine specific kinases. By Northern blot hybridization we have determined that multiple transcripts hybridize with the sik catalytic domain and cDNA clones encoding these transcripts have been isolated and are currently being characterized. In addition to the intestine, we detect high levels of sik expression in the skin and tongue. The tissues expressing sik are functionally dependent on their epithelial linings that are constantly regenerating, migrating, and differentiating. It is possible that the sik kinase plays a role in the regulation of one of these processes.

THE C-TERMINUS OF THE GAMMA SUBUNIT OF ROD cGMP PDE CONTAINS DISTINCT SITES OF INTERACTION WITH THE ENZYME CATALYTIC SUBUNITS AND TRANSDUCIN ALPHA.

N.P. Skiba, N.O. Artemyev, J.S. Mills and H.E. Hamm Dept. of Physiol. & Biophys. Univ. of Illinois College of Medicine, Chicago, IL 60612.

Interaction between the GTP-bound form of the transducin α-subunit (α_t) and the γ-subunit (Pγ) of cGMP-phosphodiesterase (PDE) is a central step in effector enzyme activation during photon signal transduction of vertebrates. We have utilized four peptides of Pγ corresponding to amino acid residues 24-46, 70-87 (synthetic peptides), 46-87 (tryptic fragment) and 58-87 (CNBr-fragment) to localize regions on Pγ that are involved in the interaction with α_t. Binding of αt to Pγ was monitored by measuring the fluorescence increase of Pγ labeled with lucifer yellow vinyl sulfone (PγLY). Binding of α_t to Pγ peptides or Pγ was determined directly (using peptides labeled with the fluorescent probe) or indirectly (using competition of the peptides with PγLY for binding to α_t).

We found that α_t bound to both the central region Pγ-24-46 (Kd=0.7 μM) and the C-terminal region Pγ-46-87 (Kd=1.5 μM) with a similar affinity. Subsequent N-terminal truncation of Pγ-46-87 (Pγ-58-87, Pγ-70-87) did not significantly reduce its ability to bind αt. Peptide Pγ-70-87 also inhibited trypsin-activated PDE (tPDE) with a Ki~0.8 μM. A set of C-terminally truncated mutants of Pγ as well as mutants with amino acid substitutions targeted to the very C-terminus of Pγ and the region 70-76 were generated by site-directed mutagenesis. The functional properties of the Pγ mutants were examined in fluorescent binding assay with α_t and in inhibition of PDE activity.

Our data suggest that a relatively small C-terminal region of Pγ contains distinct sites for interaction with PDE catalytic subunits (Pγ-77-87) and with αt (Pγ-70-76). The C-terminal sequence $Gly^{85}Ile^{86}Ile^{87}$ plays critical role in Pγ binding to PDEαβ and in inhibition of enzyme catalytic activity.