GROWTH FACTOR SIGNAL TRANSDUCTION

Organizers: Lewis Cantley, Tony Hunter, Frank Mc Cormick and Michael Karin

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Ligand-Stimulated Protein-Tyrosine Kinases (joint)

D 001 RECEPTOR PROTEIN-TYROSINE KINASES AND PHOSPHATASES Tony Hunter, Bill Boyle, Rick Lindberg, David Middlemas, Sharon Tracy, Peter van der Geer, and Jim Woodgett. The Salk Institute, P.O. Box 85800, San Diego, CA 92186

We have identified 2 novel receptor-like PTKs by screening cDNA libraries with oligonucleotide probes to consensus sequences. One of these, *eck*, isolated as a HeLa cell cDNA, is mainly expressed in tissues containing proliferating epithelial cells (skin, lung and intestine). The *eck* protein is a member of a small family of receptor PTKs, which includes *eph, elk* and *eek*. The *eck* protein is phosphorylated on Tyr in immunoprecipitates from epithelial cell lines, showing that the *eck* protein is a PTK. Staining of tissue sections shows that *eck* is expressed in epithelial cells in kidney and in small intestine. A second putative PTK, *trk*B, was isolated from a rat cerebellar library. The *trk*B protein is closely related to but distinct from the *trk* receptor-like PTK. *trk*B is primarily expressed in brain, as a series of RNAs ranging from ~1-13 kb. The smaller RNAs are too short to encode the intact protein. Analysis of additional *trk*B cDNAs indicates that there are mRNAs encoding 2 different truncated forms of *trk*B, which are both truncated just downstream of the TMD, and have short distinct C-termini. We are investigating the distribution of the 3 types of *trk*B mRNA and their protein products in the brain. We have identified Y706 lying in the kinase insert as a major autophosphorylation site in the murine CSF-1 receptor, and mutated Y706 and Y807 in this receptor to F or G. When expressed in Rat-2 cells the F807 and G706 mutant receptors respond to CSF-1, but significantly less well than wt receptors. G807 mutant receptors lack PTK activity and fail to respond to CSF-1. F807 receptors have 2-3 fold reduced PTK activity in vitro and cells expressing F807 receptors respond less well to CSF-1 than cells expressing wt receptors. Using PCR we have isolated a cDNA clone for a novel receptor-like PTPase, PTP-α, from an NIH 3T3 cell cDNA library. PTP-α has a rather small extracellular domain of 141 residues, including a signal peptide, which lacks Cys, is rich in Ser and Thr, and contains several potential sites for N-linked glycosylation. Like other receptor PTPases PTP-α has twin catalytic domains, and when expressed in E. coli the cytoplasmic domain has PTPase activity against a variety of peptide substrates. PTP-α is widely expressed in rat tissues as a 2.8 kb mRNA. Using antisera against recombinant PTP-α we are studying the biosynthesis and phosphorylation of PTP-α.

To determine how PKs activated at the cell surface induce nuclear events, we are examining the phosphorylation of nuclear regulatory proteins. The transcription factor cJun, which associates with cFos and binds to the TPA-response element (TRE) upstream of genes induced upon activation of PKC, is a phosphoprotein with 5 major sites of Ser and Thr phosphorylation. Three of these show decreased phosphorylation upon TPA treatment, and are clustered just upstream of the basic region and leucine zipper, which form the minimal DNA binding domain. Phosphorylation of these sites in vitro by glycogen synthase kinase 3 (GSK-3) in recombinant c-Jun protein decreases its ability to bind to a collagenase TRE. We propose that cJun function is negatively regulated by phosphorylation, and that TPA activation of transcription from TRE-dependent genes may in part involve dephosphorylation of cJun. The vJun protein has a S to F change at the position equivalent to 243 in human cJun, which is one of the cJun phosphorylation sites. A F243 mutant cJun is hypophosphorylated at all 3 GSK-3 sites when transiently expressed in rat embryo fibroblasts, and shows about 10-fold greater ability to activate transcription of a TRE/CAT reporter gene than wild type cJun. The protein kinase that phosphorylates cJun in vivo is not known, but the finding that GSK-3β is the homologue of the Drosophila segment-polarity gene *zeste-white3* suggests that GSK-3 itself may be the protein kinase. We are currently investigating whether these phosphorylations affect dimerization of cJun or the ability of the dimer to bind to the TRE. We are also trying to determine whether TPA inhibits the GSK-3 like protein kinase, or else activates a protein phosphatase specific for these phosphorylation sites.

Oncogenes

D 002 BIOCHEMICAL SIMILARITIES AND DIFFERENCES BETWEEN *ras* p21 GAP AND NF1 PROTEIN, George A. Martin, David Viskochil, Gideon Bollag, Peter C. McCabe, Walter J. Crosier, Heinz Haubruck, Leah Conroy, Robin Clark, Peter O'Connell, Michael A. Innis, Ray White and Frank McCormick, Cetus Corporation, 1400 53rd Street, Emeryville, CA 94608

The neurofibromatosis type 1 (NF1) gene encodes a protein with significant sequence homology to *ras* p21 GAP, a protein that regulates *ras* function converting the active form of p21 (GTP-bound) to the inactive form (GDP-bound). We have expressed the region of the NF1 gene that is related to GAP in baculovirus and yeast vectors. This fragment accelerates the GTPase activity of human N-ras p21 in a manner that resembles the activity of GAP. However, the interaction between NF1 and *ras* differs from the interaction between GAP and *ras* in a number of ways. Most strikingly, NF1 binds to *ras* p21 with 20-times greater affinity than GAP binds to *ras* p21. This raises the possibility that in cells transformed with activated *ras* oncogenes, NF1 is a preferred target of *ras* interaction. Other biochemical differences between NF1 and GAP will be discussed in this context. Expression of NF1 in yeast showed that this protein can replace the function of the IRA genes, and down regulate yeast RAS function. In this respect, NF1 resembles human GAP. Sequences of GAP (and by inference NF1) involved in stimulating *ras* p21 GTPase have been identified using a genetic screen in yeast. Details of this scheme will be presented.

D 003 SH2 DOMAINS REGULATE THE INTERACTIONS OF PROTEIN-TYROSINE KINASES WITH THEIR TARGETS.

Tony Pawson, C. Anne Koch, Deborah Anderson, Michael F. Moran and Christine Ellis, Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, M5G 1X5, CANADA.

A number of cytoplasmic signaling proteins, including Src, Ras GTPase activating protein (GAP), phospholipase C (PLC)-γ, and Crk contain one or two copies of the non-catalytic Src homology (SH) 2 domain. Src, GAP and PLC-γ1 bind to, and are phosphorylated by, activated growth factor receptor *in vivo*. The formation of these complexes with activated EGF- or PDGF-receptors can be mimicked *in vitro* using individual SH2 domains synthesized in bacteria. The two SH2 domains of GAP and PLC-γ1 apparently bind synergistically to activated receptors. SH2 domains therefore provide a common mechanism by which diverse regulatory proteins can associate with activated receptors, and thereby couple growth factor stimulation to intracellular signal transduction pathways. We suggest that receptor autophosphorylation modulates the formation of high affinity complexes with SH2-containing proteins.

In addition to autophosphorylated growth factor receptors, the SH2 domains of Src, Crk and GAP bind to a common set of cytoplasmic tyrosine phosphorylated proteins, which may be important in transformation by oncogenic tyrosine kinases. GAP, in particular, forms distinct complexes with phosphoproteins of 62 kDa and 190 kDa in v-*src*-transformed cells. The identities and functions of these proteins are under investigation.

D 004 HOW VIRAL ONCOGENES FUNCTION IN SIGNAL TRANSDUCTION

Brian Druker, Leona Ling, David Pallas, Lindsay Frazier, Lewis Cantley#, Brian Schaffhausen,*.and Thomas M. Roberts Division of Cellular and Molecular Biology,Dana Farber Cancer Institute and Harvard Medical School, and Departments of Physiology# and Biochemistry*, Tufts Medical School, Boston Ma.

The early region of polyoma virus encodes three proteins, designated by size as the small(22kd), middle(56kd) and large(100kd) T antigens. Of these, middle T antigen(MTag) is the primary transforming protein. To date no intrinsic biochemical activity has been assigned to MTag. Instead the protein appears to function by binding to and modifying the activities of a large number of host cell proteins. The first of these proteins to be identified was the cellular tyrosine kinase, $pp60^{c-src}$(Courtneidge and Smith,1984), which is activated roughly 50 fold by MTag. However, studies of mutations in MTag revealed that activation of $pp60^{c-src}$, although necessary, was not sufficient for transformation. Our laboratories are engaged in the dual processes of using biochemical techniques to identify the other proteins and biochemical activities bound by MTag, and using genetic analyses to determine the relative role of each bound protein in the process of transformation. What we have also realized is that the proteins targeted for modification by MTag are key players in normal cellular signal transduction. The first of these signal transducers which we identified was the type 1 phosphoinositide kinase. This lipid kinase, which phosphorylates the 3'position of the inositol ring, is activated both by MTag, and, as shown by us and more recently a large number of laboratories, by a large number of growth factor receptors after ligand binding. An 85kd protein in MTag immunoprecipitates forms at least part of this enzyme. More recently we have demonstrated that two proteins of 36kd and 63 kd which bind to MTag, and to the small t antigen, represent the catalytic and a regulatory subunit of protein phosphatase 2A. PP2A is known to have a regulatory function in the cell cycle. This lecture will be centered on new information concerning these and certain of the other proteins bound to MTag, and on our attempts to uncover the defect(s) in a new class of mutations in MTag which we have found. These mutations define a motif in MTag , designated NPXY after the work of the laboratories of Brown and Goldstein, who showed that mutations in a similar motif in LDL receptor affected cellular trafficking of the receptor. Interestingly MTags mutant in this domain retain all of the known associated proteins and activities when assayed in vitro. However they are transformation defective. It now appears that one or more of the MTag associated activities are defective,when assayed in vivo in cells expressing the NPXY mutants.. Taken together these results suggest that both the exact nature of the proteins found in complex with MTag and, perhaps, the exact cellular location of the complex are key to transformation.

D 005 MOS PROTO-ONCOGENE FUNCTION, George F. Vande Woude, Ira Daar, Marianne Oskarsson, Nelson Yew, and Renping Zhou, ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702

We have been trying to determine mos proto-oncogene function since we fully expect that this will reveal how this product induces neoplastic transformation. In oocytes, maturation promoting factor (MPF) is a cytoplasmic activity that causes prophase-arrested oocytes to resume meiosis and undergo maturation. MPF is accepted as the major trigger of meiosis and mitosis in eukaryotes and has been shown to consist of at least two components, the universal (from yeast to man) cell cycle regulatory product, $p34^{cdc2}$, and a protein which appears during G2/M transition and disappears rapidly at the end of mitosis referred to as cyclin. We have presented evidence qualifying $p39^{mos}$ as an "initiator" of maturation. We have also demonstrated that loss of mos function, by using antisense mos oligodeoxyribonucleotides, causes a block in meiotic maturation both in mouse and Xenopus oocytes. These studies demonstrate the requirement of mos for oocyte maturation. More recently, it has been shown that the mos product accumulates during maturation and is stabilized in unfertilized eggs. However, after fertilization the mos protein rapidly and selectively undergoes proteolysis. Furthermore, the c-mos product has been equated with the long known cytostatic factor (CSF), an activity which is believed to be responsible for the arrest of vertebrate oocytes at metaphase II and is an apparent stabilizer of MPF. How the mos product stabilizes MPF is currently under investigation. We have proposed that the activity of c-mos as an oncogene occurs by stabilizing M-phase activities during the interphase of the somatic cell cycle. More, recently, we have demonstrated that other oncogenes also have M phase activity. We speculate that oncogenes which morhpologically transform cells may do so by expressing M-phase events during interphase. Research sponsored by the National Cancer Institute, DHHS, under contract No. NO1-CO-74101 with ABL.

D 006 THE RAS SIGNALLING PATHWAY IN YEAST: Anne Vojtek, Jeffrey Field, Jeffrey Gerst, and Michael Wigler. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

RAS proteins are highly conserved in evolution. Indeed, the yeast and vertebrate proteins are functionally interchangeable. In the yeast Saccharomyces cerevisiae, RAS activates adenylate cyclase.

We have identified a novel S. cerevisiae protein, which we call CAP, for cyclase associated protein, that is required for RAS activation of adenylate cyclase. The amino-termial domain of CAP is required for this activation. In addition, a disruption of the CAP gene in yeast has a number of other interesting phenotypes not obviously related to the RAS/cyclase pathway. These phenotypes include inability to grow on rich media, temperature sensitivity on synthetic media, sensitivity to nitrogen starvation, and sensitivity to high concentrations of valine. The carboxy-terminal domain of CAP mediates these cyclase-independent functions. We have isolated genes that in high copy suppress the inability of a cap disruption strain to grow on rich media. Their characterization suggests a role for CAP in regulation of the actin cytoskeleton. The roles of RAS and CAP in both signalling pathways will be discussed.

G Protein-Linked Receptors (joint)

D 007 REGULATION OF THE CELL CYCLE BY A NEGATIVE GROWTH FACTOR IN YEAST: *FAR1* IS AN INHIBITOR OF A G1 CYCLIN, Fred Chang and Ira Herskowitz, Department of Biochemistry and Biophysics, UCSF, San Francisco, CA 94143-0448.

We are interested in how a mating factor of *Saccharomyces cerevisiae*, α-factor, arrests the cell cycle in G1 at START. The α-factor response pathway consists of a cascade of events which involve a G-protein coupled receptor, protein kinases, a transcription factor, and "arrest" genes such as *FAR1*, which culminate in the inhibition of three G1 cyclin genes to cause cell cycle arrest. We have identified a gene *FAR1* (*f*actor *ar*rest), which is necessary for cell cycle arrest but not for other responses to α-factor, such as morphological or transcriptional induction responses. A deletion allele of *FAR1* is defective in α-factor arrest, and is not affected in mitotic division or other START controls, suggesting that *FAR1* does not have a role in more general cell cycle regulation. The nucleotide sequence of *FAR1* reveals no homologies with known proteins. *FAR1* mRNA levels are induced upon exposure to α-factor. We propose that *FAR1* is a component at the end of the α-factor response pathway which acts to arrest the cell cycle.

Genetic evidence demonstrates that *FAR1* acts to inhibit specifically one of the three G1 cyclins,*CLN2* : a *far1 cln2* double mutant arrests in response to α-factor. Because the other G1 cyclins, *CLN1* and *CLN3*, can functionally substitute for *CLN2*, other effectors must inhibit these products. A drop in *CLN2* mRNA levels upon exposure to α-factor is dependent on *FAR1*. We propose that *FAR1* acts to repress *CLN2* mRNA levels, either on the level of transcription or mRNA stability.

D 008 POSITIVE AND NEGATIVE REGULATION OF THE MATING SIGNAL TRANSDUCTION PATHWAY IN YEAST. H. G. Dohlman, J. Thorner (Dept. of Molecular and Cell Biology, Univ. California, Berkeley, CA 94720) Klim King, Marc G. Caron, and Robert J. Lefkowitz (Depts. of Biochemistry, Cell Biology, and Medicine, Duke University, Durham, NC 27710)

Eukaryotic microorganisms, such as the yeast S.cerevisiae, have proven useful for the study of G protein-mediated signal transduction processes analogous to those in animal cells. Evolutionary conservation of function has in some instances allowed substitution by mammalian genes for the corresponding genes in yeast. To facilitate functional and mechanistic studies of receptor/G protein interaction, high level expression of the human β_2-adrenergic receptor (βAR) was achieved; βAR expressed in yeast displayed characteristic ligand binding affinities, specificity, and stereoselectivity. In yeast cells lacking the endogenous G protein α subunit (GPA1 gene product), partial activation of the yeast pheromone response pathway by adrenergic agonists was observed in cells coexpressing βAR and a mammalian G protein α subunit. Hence, the mammalian receptor and $G_s\alpha$ subunit can couple to each other and to downstream effectors when expressed in yeast. This in vivo reconstitution system will be useful for examining many aspects of the ligand binding and G protein activating functions of the βAR and other cell surface receptors. Similarly, the role of negative regulatory components identified genetically in yeast is being examined in mammalian cells. For example, the yeast SST2 gene product plays a critical role in recovery from pheromone-induced responses. The mechanism of SST2 action is poorly understood, but homologous gene products may play a role in desensitization of G protein-mediated signaling in mammals as well.

D 009 MOLECULAR ANALYSES OF β-ADRENERGIC REGULATION, W.P. Hausdorff, J.A. Pitcher, J. Ostrowski, M.G. Caron, and R.J. Lefkowitz, HHMI, Depts. of Medicine and Cell Biology, Duke Univ. Medical Center, Durham, NC 27710.
Continuous exposure of cells expressing β_2-adrenergic receptors (β_2AR) to epinephrine results in a rapid attenuation of the adenylyl cyclase response. Mutation of the carboxyl terminal tail of β_2AR has previously been shown to impair a variety of regulatory processes affecting β_2AR, including agonist-induced phosphorylation of the receptor, the rapid (and reversible) loss of hydrophilic ligand binding proposed to reflect sequestration of the receptor away from the plasma membrane, and a long-term loss of all ligand binding to β_2AR (down-regulation). We describe a series of substitution mutations in the proximal portion of this domain of the receptor with selective effects on the rapid regulatory processes. Although phosphorylation of β_2AR is required for rapid desensitization, these findings indicate that neither β_2AR phosphorylation nor sequestration is a prerequisite for long term down regulation of β_2AR. In addition, these results identify a 10 amino acid stretch of the carboxyl terminal domain of β_2AR that is involved in rapid regulation of the receptor.

PI Signalling/Protein Kinase C

D 010 ONCOGENES, GROWTH FACTORS AND THE PHOSPHATIDYLINOSITOL 3-P PATHWAY, Lewis Cantley, Christopher Carpenter, Kurt Auger, Rosanna Kapeller, Brian Duckworth, Lyuba Varticovski, Stephen Soltoff, and Andrea Graziani. Dept. of Physiology, Tufts University School of Medicine, Boston MA 02111
Phosphatidylinositol 3-kinase was discovered because of its co-purification with activated protein-tyrosine kinases. This finding unveiled a new inositol lipid signalling pathway distinct from the cannonical PI turnover pathway. PtdInd 3-kinase has been purified to homogeneity from rat liver and shown to be a heterodimer of 110,00 and 85,000 Da proteins. The 85,000 Da protein directly associates with polyoma middle t. In addition, it is phosphorylated on both tyrosine and serine residues in cells transformed by middle t or stimulated by PDGF, and a host of other factors that activate protein-tyrosine kinases. The purified enzyme will phosphorylate the D-3 position of PtdIns, PtdIns(4)P and PtdIns(4,5)P_2 to produce PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 respectively. All three lipid products are detected in transformed cells: the time course for appearance of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 suggests that they may act as growth signals. The discovery of a new enzyme in platelets (Yamamoto, Graziani, Carpenter, Cantley and Lapetina) and red cells (Graziani, Ling, Endemann, Carpenter and Cantley) that converts PtdIns(3)P to PtdIns(3,4)P_2 indicates the existence of two different pathways for producing PtdIns(3,4)P_2. The mechanism by which oncogenes and growth factors activate these pathways will be discussed. The possible physiological relevance of these lipids will also be discussed.

D 011 MITOGENIC SIGNALING BY LYSOPHOSPHATIDIC ACID, Wouter H. Moolenaar, Emile J. van Corven, Kees Jalink, Thomas Eichholtz, Gijs Verheijden, Rob van der Bend and Wim van Blitterswijk, Division of Cellular Biochemistry, The Netherlands Cancer Institute, 1066 CX Amsterdam, The Netherlands.
Lysophosphatidic acid (LPA, 1-acyl-glycerol-3-phosphate), the simplest naturally occurring phospholipid, has growth factor-like properties for various cell types, including fibroblasts and epithelial cells but not lymphoid cells. In quiescent fibroblasts, LPA-induced DNA synthesis is blocked by pertussis toxin which ADP-ribosylates at least two distinct G_i-like proteins. LPA initiates the following signaling cascades: (1) GTP-dependent phosphoinositide hydrolysis (insensitive to pertussis toxin); (2) breakdown of phosphatidylcholine, involving phospholipase D activity; (3) release of arachidonic acid in a GTP-dependent manner, but independent of prior phosphoinositide hydrolysis; and (4) pertussis toxin-sensitive inhibition of cAMP accumulation. When added to selected target cells, LPA also causes rapid cell rounding within a few minutes. LPA-induced cell rounding is accompanied by protein phosphorylations, is prevented by kinase inhibitors and elevated $[Ca^{2+}]_i$, and seems not to be mediated by the afore-mentioned second messenger systems. All cellular responses are specific for LPA and independent of any cytotoxic or lytic effect. Radiolabeled LPA is partially hydrolyzed to yield mono-acylglycerol, but LPA breakdown products have no apparent mitogenic activity. Neuropeptides, such as bradykinin and endothelin, mimic LPA in evoking phospholipid hydrolysis and releasing arachidonic acid, but they fail to reduce cAMP accumulation, to induce cell rounding, and to stimulate DNA synthesis.

Taken together, our results suggest that LPA-induced DNA synthesis requires early activation of a pertussis toxin-sensitive G_i-protein mediating inhibition of adenylate cyclase, and that phospholipid hydrolysis alone is an inadequate mitogenic stimulus; they further suggest that as yet unidentified effectors are required to explain all aspects of LPA action, including stimulation of DNA synthesis. One of these effectors may function in the control of cytoskeletal organization.

D 012 ACTIVATION MECHANISM OF PHOSPHOLIPASE C ISOZYMES, Sue Goo Rhee, Ha Kun Kim, Jae Won Kim, and Do Joon Park, Laboratory of Biochemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892
In a variety of cells, receptor-mediated activation of phosphoinositide-specific phospholipase C (PLC) hydrolyzes $PtdIns(4,5)P_2$ to yield intracellular messengers, diacylglycerol, and inositol phosphates. Mammalian tissues contain at least three immunologically distinct PLC isozymes--PLC-ß, PLC-γ, and PLC-δ. It appears that there are two distinct mechanisms by which PLC can be activated: One via a guanidine nucleotide binding protein and the other through phosphorylation by protein tyrosine kinase. Stimulation of phosphoinositide hydrolysis by epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and nerve growth factor (NGF) in their target cells is accompanied by a phosphorylation of PLC-γ, but not of PLC-ß and PLC-δ. The phosphorylation occurs on both serine and tyrosine residues. Intrinsic tyrosine kinase activity of EGF and PDGF receptors is directly responsible for the phosphorylation at tyrosine residues 771, 783, and 1254 of PLC-γ. However, the NGF receptor is known not to have such an intrinsic tyrosine kinase activity. Therefore, a nonreceptor tyrosine kinase appears to be associated with the NGF receptor. The role of tyrosine phosphorylation was investigated by substituting phenylalanine for tyrosine at phosphorylation sites 771, 783, and 1254 of PLC–γ and expressing the mutant enzymes in NIH 3T3 cells. Phenylalanine substitution at tyrosine 783 completely blocked the activation of PLC by PDGF, whereas mutation at tyrosine 1254 inhibited the response by 40% and mutation at tyrosine 771 enhanced the response by 50%. Like the wild type, PLC–γ substituted with phenylalanine at tyrosine 783 became associated with the PDGF receptor and underwent phosphorylation at serine residues in response to PDGF. These results suggest that PLC–γ is the isozyme of PLC that mediates PDGF–induced inositol phospholipid hydrolysis and that phosphorylation on tyrosine 783 is essential for PLC-γ activation.

Early Genes in Growth Response

D 013 COORDINATE INDUCTION OF TRANSCRIPTION FACTORS DURING THE G_0 TO G_1 TRANSITION IN MOUSE FIBROBLASTS, R.-P. Ryseck, K. Kovary, T. Noguchi, P. Lazo and R. Bravo, Department of Molecular Biology, Bristol-Myers Squibb Institute for Pharmaceutical Research, P. O. Box 4000, Princton, NJ 08543

The expression of several genes is rapidly induced during G_0 to G_1 transition in fibroblasts, including the different members of the *jun* and *fos* families. Immunoprecipitation analyses have shown that the synthesis of c-JUN, JUN B and JUN D proteins rapidly increases following stimulation of Swiss 3T3 cells remaining at a significant level of expression for at least 8 h. JUN B protein presents the highest level of expression, 3- to 5-fold higher than c-JUN and JUN D. On the other hand, the synthesis of FOS B as for c-FOS is very transient, returning to basal levels at 2 h following stimulation. Heterodimers between c-FOS or FOS B with the different JUN proteins exist for several hours after stimulation. *In vitro* studies demonstrate that both c-FOS and FOS B associate with c-JUN, JUN B and JUN D with similar kinetics suggesting that the affinity between these proteins is alike and that the proportion of these complexes *in vivo* is possibly governed by the ratio between the different proteins. Comparison of the *in vitro* binding properties of the different JUN proteins reveals that the order of their affinities for an AP-1 consensus sequence is c-JUN > JUN D > JUN B. In all cases the presence of FOS proteins significantly increases the binding affinity of the JUN proteins.

We have demonstrated that the *fos* B gene gives raise to two protein products presenting an identical kinetic of induction following serum stimulation. Studies on the genomic structure of the *fos* B gene reveal that it is very similar to the c-*fos* gene, containing 4 exons and 3 introns. The two FOS B proteins (338aa and 236aa) are originated by an alternative splicing in exon 4. Both FOS B proteins form complexes with the JUN proteins *in vivo*. The functional activity of these proteins will be discussed.

D 014 REGULATION OF TRANSCRIPTION BY FOS AND JUN. T. Curran. Department of Molecular Oncology & Virology, Roche Institute of Molecular Biology, Nutley, NJ 07110

The proto-oncogenes *fos* and *jun* encode proteins (Fos and Jun, respectively) that participate in a heterodimeric protein complex through a leucine zipper structure that binds to AP-1 and CRE sequences. The leucine zippers involve a parallel association of α helices and are best described as a special form of the coiled-coil. Dimerization brings into juxtaposition regions of each protein rich in basic amino acids that form a bipartite DNA-binding domain. Interestingly, the DNA-binding domain adopts an α helical conformation in the presence of AP-1 sequences. Thus, DNA-bound Fos-Jun complexes could be distinguished inside the cell by alternate configurations. An unusual mechanism for regulation of Fos-Jun DNA-binding activity has been uncovered. A conserved *cys* residue in the DNA-binding domains of Fos and Jun controls DNA binding by a reduction-oxidation (redox) mechanism. Oxidation, which occurs readily even in the presence of 1 mM DTT, severely reduces DNA-binding activity. The oxidation product is not yet known but it does not involve a disulphide bond. A nuclear factor has been identified that reduces Fos and Jun causing a dramatic stimulation of DNA-binding activity. Thus, redox regulation might be involved in selecting among the many protein complexes capable of binding to AP-1 sites *in vivo*. Stimulation of transcription by Fos and Jun involves multiple interacting domains. Some of these have negative and some have positive effects on transcription *in vitro*. Therefore, the overall transcriptional activity of dimeric AP-1 complexes results from a combinatorial interaction of these regions.

D 015 TRANSCRIPTION FACTORS, IRFs, IN CYTOKINE-MEDIATED CELLULAR RESPONSES, Tadatsugu Taniguchi, Hisashi Harada, Gen Yamada, Nobumasa Watanabe, Jun Sakakibara, Hitomi Yamamoto, and Takashi Fujita, Institute for Molecular and Cellular Biology, Osaka University, Suita-shi, Osaka 565, Japan.

Cytokines are generated in response to many stimuli by a variety of tissues. They act on target tissues through autocrine or paracrine mechanisms, affecting various aspects of cell growth and differentiation. To date, extensive studies have been carried out on the structure and function of various cytokines in the context of basic and clinical immunology. However, information has been limited on the molecular mechanism(s) underlying cytokine-mediated activation and/or inactivation of the genetic program within the nucleus of the target cells. We have been focusing on the interferon(IFN) system, in particular, on the mechanism of gene regulation operating in this system.

Interferon(IFNs), as a class of antiviral cytokines, are also known as "negative growth regulators", they inhibit the growth of a variety of normal and malignant cells. Normally, Type I IFNs (i.e. IFN-α, -β) are not induced, but viruses and a number of other cytokines transiently activate the IFN genes.

In order to elucidate the molecular mechanisms of cellular responses by viruses and cytokines, we have identified two nuclear factors, IRF-1 and IRF-2, both bind to the regulatory cis-elements of IFN and IFN-responsive genes. The genes encoding IRF-1 and IRF-2, have been cloned and extensively characterized. The IRF cDNA expression studies in factor-negative cells have revealed IRF-1 and IRF-2 to function as transcriptional activator and repressor, respectively. In normal cells, the IRF genes are subject to induction through stimuli such as viruses and cytokines including IFNs per se. The findings provide evidence for the presence of an elaborate network of cytokines system wherein the IRFs play a crucial role for the cytokine-mediated cellular responses.

As an approach to assess the role of the IFN-inducible IRF-1 gene, we generated transgenic mice carrying the human IRF-1 gene linked to the Ig heavy chain enhancer. In those mice, all the lymphoid tissues examined showed a dramatic depletion of B cells.

D 016 NUCLEAR ONCOPROTEINS AS TRANSCRIPTION FACTORS, Inder M. Verma, Lynn J. Ransone, Jun–ichiro Inoue, Lawrence D. Kerr, Rivka Ofir, V.J. Dwarki and Tony Hunter, Molecular Biology and Virology Laboratory, The Salk Institute, La Jolla, CA 92037

The immediate response of the cell to external stimulus is the induction of genes. Prominent among these early response genes are the nuclear oncogenes like Fos, Jun, Myc, Rel, etc., which can act either as transcription factors or cofactors. Protooncogenes Fos and Jun serve as a paradigm of interaction of two nuclear proteins for optimal DNA binding and transcriptional transactivation. Transcription of protooncogene Fos is autoregulated and depends on the state of phosphorylation of its own product. Interaction of Fos with Jun depends on the formation of heterodimers via the leucine zipper such that the basic domains are juxtaposed for efficient DNA binding. Mutation in the leucine zipper domain affects not only the protein-protein association, but also DNA binding. Conversely mutation in the DNA binding domain influences Fos-Jun heterodimer formation. We have generated DNA binding mutants of Jun (JunΔRk) which are functional transdominant negative mutants. F9 cells producing JunΔRk cannot be differentiated by retinoic acid.

The product of another early response gene Rel is able to bind to κB site and cause transcriptional transactivation in a sequence specific manner. In contrast, the v-Rel oncoprotein can bind to κB site but is unable to activate transcription. We will discuss the role of nuclear oncoproteins in cell growth, differentiation and development.

Lymphocyte Activation

D 017 TYROSINE PROTEIN KINASE SIGNAL TRANSDUCTION IN LYMPHOCYTES: Joseph B. Bolen, Anne L. Burkhardt, Philippe Bishop, Elisa Eiseman, Ivan D. Horak, Zhen-hong Li, and Peter A. Thompson, Laboratory of Tumor Virus Biology and Medicine Branch, National Cancer Institute, Bethesda, Maryland 20892.

The src-related tyrosine protein kinases are thought to be involved in the transduction of signals controlling cell growth as well as specialized functions in fully differentiated cells including those of hematopoietic origin. The association of one member of this family of enzymes, $p56^{lck}$, with the CD4 and CD8 surface receptors in T lymphocytes has provided a model system through which the first physiologic functions for a src-related kinase have been evaluated. Current experimental evidence indicates that the CD4/CD8-$p56^{lck}$ complexes function in concert with the T-cell antigen receptor complex to initiate signalling in a manner similar to that of receptor tyrosine protein kinases such as the PDGF receptor. In addition, $p56^{lck}$ appears to be involved with intracellular signals initiated through the IL-2 receptor. These initial observations in T lymphocytes have led us to investigate the potential structural and/or functional association of src-family members with receptor systems in other hematopoietic cell types. Evidence that members of the src family of tyrosine protein kinases are involved in mediating signalling events through diverse cellular surface ligand receptors such as the high affinity IgE receptor in basophils/mast cells and the IgM and IgD antigen receptors in B lymphocytes will be presented.

D 018 Pathways of Communication Between the T Lymphocyte Antigen Receptor and the Early Activation Genes *Gerald R. Crabtree, Katharine S. Ullman, W. Michael Flanagan and Jeffrey P. Northrop*
Stanford University Medical School, Stanford CA 94305

T lymphocytes capable of responding to virtually any antigen are produced in the thymus as a result of the processes of selection and intrathymic differentiation. These cells migrate to the peripheral lymphoid organs and respond to specific antigen by the sequential activation of several hundred genes, resulting in proliferation and immunologic function two weeks later. This autonomous program of differentiation appears to coordinate the immune response by cell-cell interactions and the sequential production of cytokines necessary for the function of B cells, macrophages and other cell types. The cellular events resulting in activation are complex and probably more similar to a developmental inductive influence than hormonal or growth factor stimulation. Activation requires the concurrent delivery of at least two signals. One of these arises from the antigen receptor-MHC interaction and requires cell-cell contact, intracellular adhesive molecules and the activation of tyrosine kinases and phosphorylases. The second signal is provided by IL-1 or IL-6. Experimentally, these two stimuli can be roughly mimicked by agents that increase intracellular Ca^{++} and activate protein kinase C. We have been attempting to understand the complex sequence of events that initiate T cell activation by studying the lines of communication between the antigen receptor and the IL-2 gene. The IL-2 gene is activated several thousand-fold within one hour of the delivery of the two signals and reflects the complexity of the cellular commitment to differentiation. The regulation of the IL-2 gene is mediated by a 250 bp transcriptional enhancer that is expressed only in T cells and is rigorously under the control of the antigen receptor. This enhancer appears to function by integrating the complex regulatory influences that initiate T cell activation by the use of transcription factors that respond to less complex signals. Ap-1, Ap-3, and NF-kB respond primarily to PMA stimulation while an Octamer binding protein and NF-AT (nuclear factor of activated T cells) require signals from the antigen receptor. NF-AT appears to be responsible for restricting the activation of IL-2 to antigen-stimulated T cells. The immunosuppressive prolyl isomerase inhibitors, cyclosporin and FK-506, which specifically and completely block T cell activation block transcriptional activation by NF-AT but have little effect on NF-kB, Ap-1, Sp-1 or CBP. Our studies suggest that NF-AT plays a role in commitment of T cells to immunologic activation; however, cloning and expression of this molecule will be essential to test this possibility.

D 019 STRUCTURE FUNCTION ANALYSIS OF THE T CELL ANTIGEN RECEPTOR, Richard D. Klausner, Cell Biology and Metabolism Branch, NICHD, NIH, Bethesda, Maryland 20892

The basis for the ability of T cell to recognize and respond to specific antigens rest with the immunoglobulin-like T cell receptor present on the surface of all mature T cells. This receptor is a hetero-oligimer composed of at least six different subunits: two clonotypic chains form a heterodimer responsible for antigen recognition, three CD3 chains (gamma, delta and epsilon), appear to exist in the receptor as two heterodimers (gamma-epsilon and delta-epsilon) and finally the zeta dimer. At least three different zeta dimers utilizing three zeta related proteins have been found as part of the TCR complex. In addition, zeta dimers are utilized by signalling Fc receptors in non-T cells. A very early event in signalling through the TCR is the activation of one or more non receptor tyrosine kineses. Recent evidence demonstrating the noncovalent association of the TCR with a complex of proteins containing the fyn protoncogene suggest that this may represent one of the TCR associated tyrosine kineses. How the tyrosine kinase is coupled and activated is unknown, but the possible role of tyrosine phosphatase activity in this process will be presented. Functional analysis of structural mutants of the TCR point to an essential role for the cytoplasmic tail of the zeta chain for ligand induced signalling.

Regulation of Transcription by Phosphorylation (joint)

D 020 REGULATION AND FUNCTION OF YEAST TRANSCRIPTIONAL ACTIVATOR ADR1, Clyde L. Denis, Robert C. Vallari, William J. Cook, Deborah C. Audino, Daniel L. Chase, Susan C. Fontaine, Stephen P. Mosley, and Lynne T. Bemis, Department of Biochemistry, University of New Hampshire, Durham, NH 03824

ADR1, a multiply phosphorylated protein consisting of 1323 amino acids, is required for the transcription of the glucose-repressible alcohol dehydrogenase (ADH2) from Saccharomyces cerevisiae. Several functional domains in the ADR1 protein have been identified. Two zinc-fingers in its N-terminal region are required by ADR1 to bind to the UAS1 region of ADH2. At least two other widely separated regions appear to be important for its activation of transcription. The activity of ADR1 is regulated by glucose through several different mechanisms. Glucose reduces ADR1 mRNA levels and inhibits its translation. The region involved in the translational regulation of ADR1 has been localized to the center of its coding region. In addition, ADR1 appears to be post-translationally regulated. Phosphorylation of ser-230 by cAMP-dependent protein kinase (cAPK) has been shown to inhibit ADR1 function (1). cAMP levels in yeast are known to increase in response to glucose-induced activation of the adenyl cyclase effectors CDC25 and RAS. Mutations in ADR1 that occur between residues 227 and 239 increase ADR1 activity by sixty-fold under glucose repressed conditions. Many but not all of these mutations reduce or eliminate cAPK phosphorylation of ADR1 or synthetic peptide substrates modeled on the ser-230 phosphorylation region. These results suggest that the ADR1 mutations enhance ADR1 activity by structural alterations of the 227-239 region that include but are not limited to effects on the phosphorylation state of ADR1. These alterations either allosterically activate ADR1 or affect its contact with another protein. In addition to cAPK control of ADR1, two other protein kinases, CCR1 (SNF1) and SCH9, have been found to be required for ADH2 expression. CCR1 and SCH9, however, act on factors that function independently of ADR1 to control ADH2. The mechanism by which glucose coordinates the control of these multiple protein kinases remains unclear.

(1) Cherry, J.R., Johnson, T.R., Dollard, C., Shuster, J.R., and Denis, C.L. (1989) Cyclic AMP-Dependent Protein Kinase Phosphorylates and Inactivates the Yeast Transcriptional Activator ADR1. Cell 56, 409-419.

D 021 *TRANS*-ACTIVATION BY VIRAL ONCOGENE PRODUCTS. Joseph R. Nevins, Duke University Medical Center, P.O. Box 3054, Durham, NC 27710

The analysis of transcriptional control in animal cells has been facilitated through the use of simple viral systems that encode transcriptional regulatory proteins. Perhaps the best studied of the viral *trans*-activators is the ElA gene of adenovirus. Recent studies of ElA-mediated *trans*-activation have demonstrated that multiple cellular transcription factors are likely targeted by ElA to effect an activation of transcription. At least two of these factors, E2F and E4F, are regulated via changes in DNA binding activity, dependent on a phosphorylation event. E2F DNA binding activity is further regulated through an interaction with a product of the early viral E4 gene allowing E2F to bind cooperatively and form a stable complex on the promoter. E2F can also be found in association with cellular proteins in extracts of a variety of uninfected cells. The interaction of these cellular proteins with E2F prevents E4 binding. ElA proteins are capable of dissociating E2F from these cellular complexes, allowing the E4 protein to interact. The ability of ElA to dissociate E2F from these interactions is dependent on sequences in conserved domain 1 and 2 of ElA, a region of ElA sequence that is required for oncogenic activity and that is homologous to other oncogenes such as SV40 T antigen and human papillomavirus E7. Interestingly, T ag and E7 also appear to *trans*-activate by targeting the E2F factor. Indeed, E7 is also capable of disrupting the E2F complexes, suggesting that a variety of viral *trans*-acting proteins may utilize common mechanisms for transcription factor control.

Finally, we also find that E2F activity is regulated during a cell proliferative response, consistent with the presence of E2F binding sites in a number of cellular genes regulated by cell proliferation. Moreover, the interaction of cellular proteins with E2F varies during the proliferative response indicating a regulatory role for these interactions. Since these complexes are disrupted by viral oncogene products, this activity may contribute to the alteration of cell growth control by these oncogenes.

Cell Cycle

D 022 FISSION YEAST CYCLINS, Connolly, T., Molz, L., Futcher, B. and Beach, D., Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, NY 11724. We have identified five cyclins. Three of these, typified by cdc13, are of B type. One is a G_1-cyclin. A further cyclin, encoded by the mcs2 gene, is of a new class. The role of cyclins in fission yeast will be discussed.

D 023 DIRECTING CELL DIVISION DURING DEVELOPMENT, Patrick H. O'Farrell, Bruce A.E. Edgar, Christian, F. Lehner, Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143

Several evolutionarily conserved proteins comprise a universal mitotic trigger. The timing of early embryonic cell cycles is governed by the firing of this trigger. The key component of the trigger is a mitotic kinase which is only activated upon association with a cyclin protein and removal of inhibitory phosphates. In frog eggs the early cleavage divisions are controlled, at least in part, by the periodic synthesis and destruction of cyclin proteins. However, in Drosophila embryos it appears that control is exerted mainly by control of the dephosphorylation of the mitotic kinase, $p34^{cdc2}$.

After 13 rapid and synchronous mitotic cycles the timing of the fourteenth embryonic cell cycle of Drosophila is regulated so that cells in different positions divide at different times. This pattern of divisions, which is detailed and precisely reproduced from embryo to embryo, is controlled by the timing of transcription of gene string. The string product is required for mitosis and by analogy with its homolog in yeasts (S. pombe cdc25) is needed for the dephosphorylation of $p34^{cdc2}$. Because string RNA and protein are unstable, mitosis requires concurrent string gene expression. During cell cycles 14, 15 and 16, string is transcribed in pulses at different times in different cells. string transcription precedes each mitosis by about 15 min and string RNA disappears rapidly after each division. The spatio-temporal pattern of string expression appears to be controlled by the segmentation genes that pattern the early embryo.

D 024 REGULATION OF $p34^{cdc2}$. Helen Piwnica-Worms, Laura L. Parker, Sue E. Atherton-Fessler, Margaret Lee, Scott Ogg. Department of Physiology, Tufts University School of Medicine, Boston, MA 02111. Within the past few years, tremendous advancements have been made in our understanding of how the eukaryotic cell cycle is regulated. In species as diverse as yeast and man, proteins with remarkable conservation in both structure and function have been identified. One of these key regulators is a serine/threonine protein kinase known as $p34^{cdc2}$ (Simanis and Nurse, (1986) Cell 45, 261-268). The regulation of $p34^{cdc2}$ has been shown to be quite complex involving changes both in phosphorylation as well as association with other cell-cycle regulatory proteins (p13 and cyclin). Two mitotic control genes in fission yeast that may regulate $p34^{cdc2}$ by altering it's state of phosphorylation include wee1+ and cdc25+ (Russell and Nurse, (1986) Cell 45, 145-153; Russell and Nurse, (1987) Cell 45, 559-567). Genetic evidence suggests that cdc25+ encodes an activator of $p34^{cdc2}$ whereas wee1+ encodes a negative regulator. $p34^{cdc2}$ is phosphorylated on tyrosine as cells progress from the G1 to the G2/M phases of the cell cycle (Draetta et al., (1989) Nature 336, 738-744.) and is dephosphorylated on tyrosine just prior to entry into M phase (Morla et., (1989) Cell 58, 193-203). Dephosphorylation on tyrosine correlates with maximal kinase activity. The kinases and phosphatases responsible for this cyclical tyrosine phosphorylation and dephosphorylation remain undetermined. Further, the temporal ordering of events that leads to the activation of $p34^{cdc2}$ and the entry of cell into mitosis remains unknown. To determine how the tyrosine phosphorylation of $p34^{cdc2}$ is regulated, we have overproduced $p34^{cdc2}$, as well as several of the regulators mentioned above, using a baculoviral expression system. $p34^{cdc2}$ is produced as a 34Kd protein in insect cells whereas wee1+ encodes a protein of approximately 107kd. Surprisingly, immunoprecipitates of $p107^{wee1}$ possess tyrosine kinase activity and $p34^{cdc2}$ becomes phosphorylated on tyrosine when co-produced with $p107^{wee1}$ in insect cells. These results suggest a role for $p107^{wee1}$ in the regulation of $p34^{cdc2}$ by tyrosine phosphorylation. The effects of other cell-cycle regulatory proteins on the tyrosine phosphorylation of $p34^{cdc2}$ will also be discussed.

D 025 G1/S-PHASE CONTROL IN YEAST AND HUMAN CELLS, Steven I. Reed, Jayant Ghiara, Martha Hentze, Daniel Lew, Constance Stueland, Katsunori Sugimoto and Curt Wittenberg, Department of Molecular Biology MB-7, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, CA 92037.

The *S. cerevisiae* gene *CDC28* encodes a 34 kilodalton protein kinase catalytic subunit with highly conserved couterparts in all eukaryotic organisms. In both *S. cerevisiae* and fission yeast, *S. pombe*, genetic analysis indicates a role for this protein kinase, p34, both in regulating the G1 to S-phase transition and the G2 to M-phase transition. In *S. cerevisiae* our investigations suggest that these diverse regulatory activities are achieved by assembling active heteromultimeric complexes with G1- and G2-specific subunits. G1 regulation depends upon accumulation of a class of unstable cyclin-like proteins that are essential for the assembly of active Cdc28 protein kinase complexes. These proteins (*G1 cyclins*), designated Cln1, Cln2 and Cln3, normally accumulate late in G1 and are prevented from accumulating by signals that restrain cell division in G1. Furthermore, mutational analysis suggests that the accumulation of these proteins is rate limiting for Cdc28 protein kinase activity and passage through G1. Passage through G2 depends upon accumulation of three different cyclins, Scb1, Scb2 and Scb3, that show a high degree of similarity to cyclin B, and assembly of a different Cdc28 protein kinase complex. In this case, however, cyclin accumulation, although periodic, is not rate limiting. The action of a regulatory network that controls the kinase by phosphorylation and dephosphorylation of the catalytic subunit appears to be rate limiting for activity in G2. This is analogous to the mitotic regulation of p34 and cyclins in animal cells. In order to address the issue of whether G1 cyclins and p34 control the G1/S-phase transition in animal cells, human cDNAs that can rescue deletion of the G1 cyclin genes in yeast were isolated. These are currently being characterized.

Negative Regulation of Growth

D 026 FUNCTIONAL ANALYSIS OF THE RETINOBLASTOMA GENE PRODUCT
D.M.Livingston, W. Kaelin, J. DeCaprio, J. Ludlow, Z. Arany. Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA 02115.

The product of the RB-1 locus is a 928 aa nuclear, DNA-binding protein that can be identified in two generic forms-un(der)phosphorylated (pRB) and phosphorylated (pRBphos). The state of phosphorylation appears to modulate how it operates in the performance of its growth regulating action(s). There is evidence implying that pRB can help to regulate exit from the G1 phase of the cell cycle and that binding of the SV40 large T antigen (T) or phosphorylation contribute to the neutralization of this function. Major questions at present are how pRB operates biochemically and how this behavior is translated into its proposed cell cycle regulatory function.

Within the pRB sequence is a ~400 aa segment which, as a unit, can bind, specifically, to T or E1A or peptide replicas of the sequences within T and E1A which are responsible for RB binding. This domain is an established site for mutation in naturally occurring human tumors which lack RB-1 function. Such mutations have been shown to inactivate the T/E1A binding function of pRB. With this information as background, we have begun a search for cellular proteins which can specifically bind to this domain. Using an affinity chromatographic approach, a family of ~7 cell-encoded proteins which can interact with the T/E1A binding segment of pRB have been identified. None interact with any of a series of mutant domains which lack T binding function. A peptide replica of the pRB binding sequence of T (aa 102-115), which can compete with T for pRB binding, competed effectively with all of them for binding to the domain. A mutant version of that peptide which failed to compete with authentic T for pRB binding was ineffective. The latter is a copy of the 102-115 sequence contained within a non- transforming T mutant (K1) that cannot bind to pRB. Thus, cells contain proteins which can, at least in part, mimic T or E1A in their ability to bind, specifically, to the RB product. The identity of these proteins, whether they exist as single elements or complexes, and the significance of their binding to pRB are under investigation.

D 027 TRANSFORMING GROWTH FACTOR-ß AND GROWTH INHIBITION.
Joan Massagué, Cell Biology and Genetics Program & Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

The members of the TGF-ß family are secretory dimeric polypeptide that inhibit growth in multiple cell types. Biochemically, the growth inhibitory action of TGF-ß is linked to its ability to retain the retinoblastoma gene product (RB) in its unphosphorylated, growth suppressive state. In addition, TGF-ßs affect the expression of various genes encoding transcription factors (c-jun, JunB, myogenin, MyoD1) and extracellular matrix proteins. Mediation of these responses is linked to the interaction of TGF-ß with receptors I (53 kd) and II (70 kd), two cell surface proteins that bind TGF-ß with high affinity. Selective loss of either receptor type correlates with loss of TGF-ß responses. Reconstitution of expression of both receptor components in somatic cell hybrids leads to recovery of full responsiveness to TGF-ß. Evidence indicates that receptors I and II can function as components of a receptor complex that mediates multiple responses to TGF-ß. The various TGF-ß isoforms differ in their ability to bind to receptors I and II. These differences correlate with marked cell-specific differences in responsiveness to the various TGF-ß isoforms. The interaction of a given TGF-ß isoform with a limited subset of receptors dictates in a cell-specific manner the range of responses elicited by this isoform. A further dissection of the TGF-ß signal transduction system can be obtained with the use of cells that express the SV40 virus transforming protein, large T antigen (T). T binds unphosphorylated RB pertubing its growth-suppressive action and rendering cells resistant to growth inhibition by TGF-ß. However, T does not block the ability of TGF-ß to regulate expression of JunB or extracellular matrix proteins. Additional details of the receptors and events involved in the signaling of growth inhibition will be presented at the meeting.

Regulation of Transcription by Second Messengers (joint)

D 028 MULTIPLE REGULATION OF THE NF-κB TRANSCRIPTION FACTOR BY PROTEIN SUBUNITS, Patrick A. Baeuerle, Egenhart Link, Lienhard Schmitz, Ralf Schreck, Manuela B. Urban and Ulrike Zabel, Laboratory of Molecular Biology and Biochemistry, Gene Center, Ludwig-Maximilian-University, D-8033 Martinsried, West Germany.

In many cell types, the NF-κB transcription activator is involved in the rapid induction of gene expression upon stimulation of cells with agents such as viruses, parasites and cytokines. Most of its known target genes encode cytokines, immunoreceptors and acute phase proteins. The NF-κB system comprises three protein subunits: p50, the DNA binding subunit, p65, a non DNA-binding accessory protein of p50, and IκB, the inhibitory subunit. The inactive cytoplasmic form of NF-κB is composed of p50, p65 and IκB. Upon stimulation of cells, IκB is released and allows association of two p50-p65 dimers to a heterotetrameric complex. The heterotetramer is the active form of NF-κB found in nuclei. While in the inactive cytoplasmic form p65 serves to bind IκB, it has a modulatory role for the DNA binding of p50 in the nucleus. If p65 is bound to p50, κB motifs of low symmetry are preferred binding sites, the DNA bending induced by p50 is increased and the position of the bending center is shifted. In addition p65 might help to dimerize p50, to provide a transcription activation domain and to serve as receptor for IκB. The latter would allow inactivation of NF-κB and a subsequent transport of the factor back to the cytoplasm. Novel functional aspects of the NF-κB system and a more detailed characterization of the protein subunits will be presented.

D 029 POST-TRANSCRIPTIONAL AND TRANSCRIPTIONAL CONTROL OF AP-1 (JUN/FOS) ACTIVITY, Michael Karin, Tod Smeal, Bernard Binetruy, Hsin-Fang Yang-Yen, Jean-Claude Chambard, Tiliang Deng and Adriana Radler-Pohl, Department of Pharmacology, School of Medicine, M-036, University of California San Diego, La Jolla, CA 92093.

AP-1 is a transcriptional activator composed of homo-and heterodimeric Jun and Jun/Fos complexes. It is involved in the activation of various target genes, such as: collagenase, stromelysin, IL2 and TGFβ1, by tumor promoters, growth factors and cytokines. In addition AP-1 activity is elevated in response to expression of transforming oncogenes including *H-ras*, *v-src*, and *v-raf* and is required for cell proliferation. AP-1 activity is subject to complex regulation both transcriptionally and post-transcriptionally. Transcriptional control determines which of the *jun* and *fos* genes is expressed at any given time in any given cell type. Therefore, transcriptional control determines the amount and composition of the AP-1 complex. Transcription of the *jun* and *fos* genes is subject to both positive and negative autoregulation and is highly inducible in response to various stimuli including those associated with cell proliferation.

AP-1 activity is also regulated at the post-transcriptional level. Both cJun and cFos are phosphoproteins that are subject to regulated phosphorylation. In the case of cJun, phosphorylation of sites near the DNA-binding domain inhibits its DNA-binding activity while dephosphorylation reverses this inhibition. It is also possible that phosphorylation of cJun on other sites increases its ability to activate transcription without affecting its DNA binding activity.

Another mechanism that modulates AP-1 activity is transcriptional interference by members of the nuclear receptor family. For example, the glucocorticoid receptor was recently shown to interact with cJun and cFos to form a protein complex that is no longer capable of binding to either the AP-1 recognition site or hormone response elements.

Late Abstracts

TRANSCRIPTIONAL CONTROL OF cAMP-INDUCIBLE GENES, Marc R. Montminy, Gustavo A. Gonzalez, and R. Scott Struthers, The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute, La Jolla, CA 92037

How are extracellular signals transduced into physiological responses? Many of these events occur through altered patterns of gene expression mediated by second messenger pathways and their associated protein kinases. The cloning of the transcription factor CREB (cAMP Regulatory Element Binding protein) and subsequent functional characterization of the clone has enabled our laboratory to elucidate mechanisms by which protein kinases govern gene transcription. The CREB protein has a cluster of phosphorylation sites, including a site for cAMP dependent protein kinase, situated between two putative glutamine rich activator domains. Furthermore, protein kinase C, CAM kinase II, glycogen synthesase kinase III and casein kinase II also phosphorylate CREB in this region, suggesting that multiple signal transduction pathways may act within the centrally located "kinase inducible domain" (KID) to regulate CREB activity. Although phosphorylation of SER 133 by protein kinase A is required for transactivation, it does not appear to be sufficient. Deletion mapping has identified regions distinct from the protein kinase A phosphorylation site that are indispensable for the transactivation activity of CREB. These motifs include the two glutamine rich regions flanking the KID. Our results suggest that CREB can activate a number of different promoters through a glutamine type activator. It is likely, therefore, that phosphorylation regulates the transcriptional activity of CREB by causing structural changes in the KID region which render the Q regions transcriptionally competent to interact with proteins of the polymerase II complex.

Growth Factor Signal Transduction

SIGNAL TRANSDUCTION BY RECEPTORS WITH TYROSINE KINASE ACTIVITY, Joseph Schlessinger, Department of Pharmacology, New York University Medical Center, New York, NY 10016 The membrane receptor of epidermal growth factor (EGF-receptor) is composed of a large extracellular ligand binding domain, a singletransmembrane region and a cytoplasmic domain, a single transmembrane region and a cytoplasmic domain containing protein tyrosine kinase activity. We have formulated an allosteric oligomerization model for activation of the catalytic properties of neighboring cytoplasmic domains. In Vitro site directed mutagenesis was used to generate various EGF-receptor mutants. Using this approach, it was shown that the kinase activity of EGF receptor is essential for signal transduction and for normal receptor trafficking, while autophosphorylation is not crucial for receptor signaling. It was also shown that EGF is able to stimulate tyrosine phosphorylation of phospholipase C-γ and EGF also induced activation of c-raf, suggesting that this enzyme may be involved in cascade of kinases initiated by tyrosine phosphorylation. The precise composition of the transmembrane domain is not essential for receptor activity, further supporting the oligomerization model for receptor activation. Binding experiments of EGF to various chicken/human EGF receptor chimera has allowed the identification of domain III of the extra-cellular domain of EGF-receptor as a major ligand binding domain. Some interactions are also provided by domain I which, together with domain III appear to constitute the binding region for EGF.

SIGNAL TRANSDUCTION BY PDGF RECEPTORS INVOLVES DIMERIZATION AND ASSOCIATION WITH SIGNALING MOLECULES, L.T. Williams, J.A. Escobedo, V.A. Fried, H. Ueno, and W.M. Kavanaugh Howard Hughes Medical Institute and Cardiovascular Research Institute, University of California San Francisco, CA 94143 and Department of Cell Biology and Anatomy, New York University, Valhalla, New York 10595
To study the role of receptor dimerization in signal transduction we have used mutants of the PDGF β receptor (PDGFR) that bind PDGF but lack intrinsic kinase activity. When co-expressed with wild type receptors, these mutants formed heterodimers with wild type PDGFR in a PDGF-dependent manner. A heterodimer consisting of wild type PDGFR and a PDGFR with a truncated tyrosine kinase domain was defective in autophosphorylation, whereas the ligand-induced homodimer of wild type receptor was autophosphorylated. When co-expressed with wild type receptors in Xenopus oocytes, the mutant PDGFR abolished signal transduction by wild type PDGFR but did not affect signal transduction by wild type FGF receptors (FGFR). A similar mutant of the FGFR blocked signal transduction by wild type FGFR but not by PDGFR. These findings show that receptor dimerization is required for PDGF receptor signal transduction, suggest that autophosphorylation is intermolecular reaction between the components of a receptor dimer and establish a unique approach to inhibit receptor function in vivo.
Autophosphorylated PDGFR associates with several cellular molecules and phosphorylates some of them on tyrosines. We have recently focused on an 85 kDa protein (P85) that associates with ligand-activated PDGFR and is one of the major tyrosine-phosphorylated proteins in PDGF-stimulated cells. We purified P85 using tyrosine-phosphorylated PDGFR as an affinity reagent and cloned the cDNA that encodes P85. P85 contains two SH2 domains but no identifiable kinase motifs. Thus P85, like GTPase activating protein, may associate with PDGF receptor through its SH2 domains. We showed that P85 binds to a 16 amino acid segment of PDGFR the that contains phosphotyrosine at position 719. This segment of PDGFR also binds phosphatidylinositol 3 kinase (PI3 kinase), an enzyme that has been implicated in the regulation of growth of both normal and transformed cells. P85 expressed in cos cells competes with PI3 kinase and 110 kDa protein for binding to the receptor. Thus P85 is either a subunit of PI3 kinase or an antagonist that competes with PI3 kinase for binding to the receptor. Thus signaling seems to involve ligand-induced receptor dimer formation, intermolecular autophosphorylation of receptors, and the binding of SH2 domain-containing proteins to autophosphorylation sites on the receptor. The SH2 domains appear to recognize phosphotyrosine in a specific sequence context.

Growth Factor Signal Transduction

Protein Phosphorylation I

D 100 PURIFIED PI 3-KINASE ASSOCIATES WITH AND IS PHOSPHORYLATED BY BACULOVIRUS EXPRESSED POLYOMA MIDDLE T ANTIGEN/pp60$^{c\text{-}src}$ COMPLEX, Kurt R. Auger, Christopher L. Carpenter, Helen Piwnica-Worms, and Lewis C. Cantley, Department of Physiology, Tufts University School of Medicine, Boston, MA 02111.

In vitro reconstitution of the polyoma virus middle T antigen (mT) mT/pp60$^{c\text{-}src}$ complex and phosphoinositide 3-kinase (PI 3-kinase) has been accomplished with immunopurified baculovirus expressed mT/pp60$^{c\text{-}src}$ and purified rat liver PI 3-kinase. The association of PI 3-kinase with the mT/pp60$^{c\text{-}src}$ complex was dependent on an active protein-tyrosine kinase: pp60$^{c\text{-}src}$ mutated in the ATP binding (lys 295 to met, mT/pp60$^{295c\text{-}src}$) still complexed with mT but was unable to bind PI 3-kinase. The associated PI 3-kinase was able to phosphorylate phosphatidylinositol, phosphatidylinositol-4-phosphate, and phosphatidylinositol-4,5-bisphosphate. The mT/pp60$^{c\text{-}src}$ complex phosphorylated both subunits of PI 3-kinase on tyrosine residues. The immunopurified mT/pp60$^{c\text{-}src}$ complex was also shown to associate with PI 3-kinase from whole cell lysates. This association was also dependent on the active tyrosine kinase activity of pp60$^{c\text{-}src}$. Comparison of immunopurified mT/pp60$^{c\text{-}src}$ and mT/pp60$^{295c\text{-}src}$ abilities to associate with proteins from whole cell lysates demonstrate that the 110 and 85 kd proteins of PI 3-kinase are the major peptides dependent on tyrosine phosphorylation for association to the complex. This *in vitro* association is a step toward analysis of protein-protein interactions important in the signal transduction pathway of oncogenic proteins.

D 101 INDUCTION OF PLASMINOGEN ACTIVATOR GENE EXPRESSION BY pp60$^{v\text{-}src}$ DEPENDS ON PROTEIN KINASE ACTIVITY AND MYRISTYLATION BUT NOT THE MAJOR SITES OF PHOSPHORYLATION S. M. Bell and J. L. Degen, Children's Hospital Research Foundation, Cincinnati, OH 45229.
Urokinase-type plasminogen activator (uPA) gene transcription and mRNA levels are increased >50-fold in chicken embryo fibroblasts (CEF) following infection by Rous sarcoma virus (RSV). Studies of conditional mutants of pp60$^{v\text{-}src}$ suggest that the modulation of uPA transcription is dependent on the protein tyrosine kinase activity of the oncogene product. A more general role of protein phosphorylation in the regulation of uPA gene expression is suggested by the findings that protein kinase C activating agents (e.g., PMA) synergistically enhance uPA gene transcription in RSV-CEF, whereas protein kinase A activating agents (e.g., 8-Br cAMP) repress induction of uPA mRNA. To define the features of pp60$^{v\text{-}src}$ critical to the modulation of uPA gene expression, we have analyzed cellular uPA mRNA levels in CEF expressing site-directed mutants of *src* . A mutant lacking catalytic activity as a result of an amino acid substitution in the ATP binding pocket (Lys_{295} to Met) was inactive in increasing uPA mRNA, confirming the role of protein tyrosine kinase activity in the regulation of gene expression. However, catalytic activity alone is insufficient to increase uPA gene transcription; a mutant of pp60$^{v\text{-}src}$ (Gly_2 to Ala) that maintains full catalytic activity, but fails to be either myristylated or localized to the plasma membrane, did not induce uPA gene expression. Elimination of the major sites of pp60$^{v\text{-}src}$ phosphorylation (Ser_{12}, Ser_{17}, and Tyr_{416}) did not diminish either the induction of uPA mRNA or the synergy with PMA. However, cells expressing a mutant lacking Tyr_{416} were refractory to the repressive action of cAMP.

D 102 **PDGF INTERRUPTION OF INTERCELLULAR COMMUNICATION IS INDEPENDENT OF RECEPTOR TYROSINE KINASE ACTIVITY. Alton L. Boynton, Seavosh Danesh, Lixin Xhang, Martha Kanemitsu and Nicholas M. Dean. Cancer Research Center of Hawaii, 1236 Lauhala St., Honolulu, Hi 96813.**

Polypeptide growth factors function through high affinity and specific plasma membrane receptors. Several of these receptors, such as the epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and colony stimulating growth factor (CSF), are believed to elicit their biological functions solely through receptor tyrosine protein kinase activity (TPK). The receptor TPK phosphorylates specific substrates that are thought to mediate the ensuing physiological response. Here we demonstrate that PDGF interruption of intercellular communication (ICC) is independent of receptor TPK activity. Incubation of C3H10T1/2 mouse fibroblasts with genistein, a TPK inhibitor, before the addition of PDGF, blocks autophosphorylation of the PDGF receptor but does not interfere with the ability of PDGF to interrupt ICC. To our knowledge, this represents the first biological end point of PDGF independent of its receptor tyrosine kinase activity.

D 103 GROWTH HORMONE STIMULATED TYROSYL PHOSPHORYLATION OF CELLULAR PROTEINS. George S. Campbell and Christin Carter-Su, Department of Physiology, University of Michigan, Ann Arbor, MI.
Growth hormone (GH) possesses diverse growth promoting and metabolic activities. While it is generally accepted that the various actions of GH stem from the interaction of GH with its plasma membrane receptor (GHR), the actual mechanisms of trans-membrane signaling utilized by the GH-GHR complex are not known. We have previously demonstrated that the binding of GH to the GHR stimulates tyrosyl phosphorylation of the GHR itself and that tyrosine protein kinase (TPK) activity copurifies with GH-GHR complexes. To investigate further the possibility that this GH stimulated TPK activity could mediate some of GH's effects, we have employed a Western blot assay, using an anti-phosphotyrosine antibody, to monitor the formation of phosphotyrosine-containing cellular proteins following exposure of 3T3-F442A fibroblasts and adipocytes to GH. We find that physiologically relevant GH doses (5-500ng/ml) produce increased levels of a phosphotyrosine-containing protein with an apparent Mr consistent with that of the GHR (pp121). We also find that increased levels of phosphotyrosine-containing pp121 can be observed as early as one minute following the addition of GH. This time course is sufficiently rapid to be consistant with the GH stimulated TPK activity being an initiating event for even the most rapid physiological responses to GH. In addition to pp121, we often see the appearance of another phosphotyrosine-containing protein. This protein, termed pp194, is only observed after prolonged (~60 minutes) exposure to GH.

D 104 DOWN-REGULATION OF THE EGF RECEPTOR DURING ADENOVIRUS INFECTION DOES NOT REQUIRE PROTEIN TYROSINE KINASE ACTIVITY, Cathleen Carlin, Patricia Hoffman, Bill Wold*, and Brian Hoffman, Department of Physiology and Biophysics, Case Western Reserve Univ, Cleveland, OH 44106, and *Molecular Virology, St. Louis Univ Med Sch, St. Louis, MO 63110
It is known that human group C adenoviruses (Ad) encode a protein of predicted MW 10,400 (10.4K) which induces internalization and degradation of the EGF receptor (EGF-R). Retrovirus-mediated gene transfer has been used to show that 10.4K elicits the same response in the absence of other viral proteins. Peptide antibodies precipitate two species, a primary translation product of MW 13.7K, and a 11.3K protein; these species have apparent pIs of 7.5 and 7.2, respectively, and form disulfide-bonded homodimers _in vivo_. Using a phosphotyrosine-specific monoclonal antibody, we have also shown that 10.4K expression does not cause EGF-R tyrosine autophosphorylation _in vivo_. Moreover, unlike what is seen with ligand-induced internalization, EGF-Rs do not undergo dimerization during Ad infection. We have also studied 10.4K-mediated receptor internalization in A431 cells, in which high concentrations of ligand actually inhibit internalization, presumably due to saturation of the endocytotic pathway. Ad infection overcomes the ligand-induced block, suggesting that 10.4K expression somehow compensates for the limiting cellular component, or that a different internalization pathway is utilized. This work was supported by NIH grant CA49540.

D 105 LARGE SCALE EGF RECEPTOR MICRO-AGGREGATION IS NOT NECESSARY FOR SIGNAL TRANSDUCTION, Kermit L. Carraway and Richard A. Cerione, Department of Biochemistry, Cell and Molecular Biology, Cornell University, Ithaca, NY 14853.
We have been studying EGF receptor-receptor interactions and the role of receptor micro-aggregation in transmembrane signaling. Using isolated plasma membranes from A431 cells, we have found that a monoclonal antibody to the extracellular domain of the EGF receptor blocks EGF-stimulated micro-aggregation without altering tyrosine kinase activity. Moreover, we have found that EGF receptor aggregation is constrained in intact cells, apparently by a cytoplasmic protein component(s). A significant proportion of this component is lost upon disruption of cells by homogenization. These data suggest that while aggregation may play a role in mitogenic signaling by the EGF receptor, large scale receptor aggregation is not necessary to transduce the signal across the plasma membrane.

D 106 THE ROLE OF TYROSINE KINASE ACTIVITY IN THE ACTIONS OF GROWTH HORMONE.
Christin Carter-Su, Department of Physiology, University of Michigan, Ann Arbor, MI 48109-0622.
Growth hormone (GH) was identified as a growth factor more than 60 years ago, yet its mechanism of action is still not understood. Over the past 8 years, my laboratory has been investigating the structure of the GH receptor and the signal transduction mechanisms by which it regulates cellular metabolism. We have used phosphotyrosyl binding antibodies combined with GH and GH receptor antibodies to show that GH promotes the phosphorylation of the GH receptor on tyrosyl residues, and that tyrosine kinase is associated with highly purified GH-GH receptor complexes. Furthermore, GH increases the amount of tyrosine kinase activity associated with the GH receptor. These results indicate that the GH receptor, or a closely associated protein, is a tyrosine kinase. The time course (within one min) and dose response (5-500 ng/ml GH) for tyrosyl phosphorylation of the GH receptor upon addition of GH to cells is consistent with tyrosyl phosphorylation being an important early event in the actions of GH. We are currently using standard biochemical techniques combined with techniques of molecular biology to determine whether the GH receptor itself or an associated protein contains the tyrosine kinase activity; to identify substrates of the GH receptor-associated tyrosine kinase; and to determine which of the diverse growth-promoting and metabolic actions of GH are dependent upon increased tyrosine kinase activity.

D 107 BIOLOGIC EFFECTS OF HER-2/*neu* PROTO-ONCOGENE OVEREXPRESSION IN HUMAN BREAST AND OVARIAN TRANSFECTED CELLS.
Victoria Chazin Campbell, Michael Kaleko, A. Dusty Miller, H. Michael Shepard, and Dennis J. Slamon, Department of Microbiology and Immunology, Department of Medicine, UCLA, CA; Department of Pediatrics, University of Washington, Seattle, WA; Fred Hutchinson Cancer Research Center, Seattle, WA; and Genentech Inc., San Francisco, CA.

The HER-2/*neu* proto-oncogene is a growth factor receptor, protein tyrosine-kinase, for which a ligand has recently been identified. The HER-2/*neu* gene is amplified and overexpressed in approximately 30% of primary breast and ovarian tumors. To assess the possible roles of amplification and overexpression of HER-2/*neu* in the pathogenesis of malignancy, and to determine the growth properties induced by ligand-stimulated tyrosine-kinase activity, multiple copies of the HER-2/*neu* gene were introduced into a variety of human breast and ovarian cell lines. Retrovirally infected clones were screened for HER-2/*neu* overexpression by immunohistochemistry and western blot analyses. Overexpression of HER-2/*neu* in these cells correlated with multiple biologic changes in both *in vitro* and *in vivo* assays. DNA synthesis and cell proliferation rates increased with increasing amounts of HER-2/*neu* expression. The cells overexpressing HER-2/*neu* also exhibited markedly different properties in response to ligand stimulation. In addition, these overexpressing cells were able to form tumors in the nude mouse whereas the parental cells could not. These data indicate that by increasing the amount of HER-2/*neu* expression in human breast and ovarian cells, we can alter some of their basic biologic growth properties, consistent with a possible pathogenetic role for this molecular alteration. Additionally, this model can be used to study signal transduction by this growth factor receptor.

D 108 EFFECTS OF *v-abl* AND PDGF RECEPTOR EXPRESSION ON DIFFERENTIATION OF THE HEMATOPOIETIC STEM CELLS, Kyunghee Choi, Gordon Keller, Department of Medicine, National Jewish Hospital, Denver, CO 80206
The early events in hematopoiesis and lymphopoiesis remain poorly understood, due to the fact that multipotential stem cells and their immediate progeny are found at extremely low frequencies in normal hematopoietic tissues. One approach towards studying these rare cell populations is to alter their growth and development through the overexpression of growth control genes. The genes we have chosen to introduce and overexpress in the primitive hematopoietic cells are a temperature sensitive (ts) mutant of the *v-abl* oncogene and the gene encoding the PDGF receptor. As a first step, we have constructed recombinant retroviral vectors that contain these genes and transfected them into the retroviral packaging cell line, GP+E. We are in the process of analyzing the transfectants to obtain high titer virus producing cell lines. These viruses will first be used to infect growth factor dependent cell lines to determine whether or not the expression of these genes will abrogate their growth factor dependence. Factor independent growth should occur only in the presence of PDGF for those cells infected with the receptor containing vriuses and only at the permissive temperature for those infected with the ts *v-abl* containing viruses. Having established that these genes do function in this fashion in these cell lines, we will next use these recombinant retroviruses to infect normal bone marrow and fetal liver cells. For most of the studies, we will use marrow populations that have been enriched for primitive stem cells. This will be accomplished by 5-fluoruracil treatment of the marrow donor mice, adherence of stem cells to tissue culture plastic, and separation by density centrifugation. In addition to studying the effect of the expression of these genes on these populations, we will also analyze the effects of their expression on the differentiation of embryonic stem (ES) cells to hematopoietic cells in culture.

D 109 Ca^{2+}- DEPENDENT PROTEOLYSIS OF LIPOCORTIN I AT A NOVEL SITE ENHANCES THE CALCIUM SENSITIVITY OF LIPOCORTIN BINDING TO PHOSPHOLIPIDS, Siew Yeam Chuah and Catherine J. Pallen, Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511.

We reported earlier that purified placental lipocortin I (LC I) is cleaved in a Ca^{2+}-dependent manner by an endogenous A431 cell membrane protease (Chuah, S.Y. & Pallen, C.J. (1989) JBC 264, 21160). Proteolysis occurs at a site N-terminal to the site of tyrosine phosphorylation (Tyr-21) and is stimulated by phosphorylation of LC I. Here we report that membrane-directed cleavage of LC I occurs at a novel site (identified by protein microsequencing) distinct from other known sites of proteolytic susceptibility and is catalyzed by a neutral protease. The Ca^{2+}-dependence of proteolysis reflects the Ca^{2+}-binding properties of LC I rather than a requirement of the protease. Cleavage does not affect the ability of LC I to inhibit phospholipase A_2 activity or to serve as a tyrosine kinase substrate. However, cleaved LC I requires a 2-fold lower concentration of Ca^{2+} for half-maximal association with phospholipid vesicles than does native LC I, demonstrating that the N-terminal region of the protein is critical in regulating properties of the C-terminal core domain and thus perhaps LC I function.

D 110 TRUNCATION MUTAGENESIS OF THE EXTRACELLULAR DOMAIN OF THE EGF RECEPTOR. Richard W. Connors and Paul R. Keller, Department of Signal Transduction, Warner-Lamber Co., Parke-Davis Pharmaceutical Research Division, 2800 Plymouth Rd., Ann Arbor, MI 48106-1047.

The extracellular domain of the EGF receptor is a complex glycoprotein containing 50 cysteine residues and 12 potential N-linked glycosylation sites. Others reported that the D3 domain contributes the majority of the EGF binding affinity through cross-linking studies and chimeric EGF receptors. Our laboratory has constructed several 'mini-genes' encoding portions of the EFGR ectodomain and analyzed their protein products after transient expression in Cos cells. We find that the carboxy-terminal half of the ectodomain is sufficient to provide folding and maintain EGF binding. We will present EGF affinities and monoclonal antibody reactivities of our truncates overexpressed in CHO cells or a baculovirus system.

D 111 EGF-STIMULATED TYROSINE PHOSPHORYLATION OF PROTEINS IN CELLS EXPRESSING EGF RECEPTORS TRUNCATED AT RESIDUE 973. Stuart Decker, Signal Transduction, Parke-Davis, 2800 Plymouth Rd., Ann Arbor, MI, 48106 and The Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48104.

EGF receptor degradation and EGF-stimulated tyrosine phosphorylation were examined in cells expressing wild-type EGF receptors (WT-EGFR) and receptors truncated at residue 973 (973-EGFR). Chronic exposure to EGF resulted in decreased steady state levels of mature receptor for cells expressing either receptor type as well as the appearance of discrete receptor degradation products. A much broader spectrum of tyrosine phosphorylated proteins was found following EGF treatment of 973-EGFR expressing cells compared to cells expressing wild-type receptors. Several additional GAP-associated tyrosine phosphorylated proteins were found in EGF-treated 973-EGFR cells relative to WT-EGFR cells. Phospholipase C-γ was phosphorylated on tyrosine residues in response to EGF treatment in cells expressing both receptor types. The data indicate that truncation of the EGF receptor at residue 973 relaxes receptor substrate specificity, perhaps contributing to the increased mitogenic potential of the 973-EGFR.

D 112 EARLY INDUCTION OF EPIDERMAL GROWTH FACTOR (EGF) RECEPTOR PHOSPHORYLATION BY TUMOR NECROSIS FACTOR (TNF) CORRELATES WITH ITS GROWTH MODULATORY PROPERTIES. N. J. Donato, D. Vijjeswarapu, M. G. Rosenblum. Dept. of Clinical Immunology, M. D. Anderson Cancer Center, Houston, Tx 77030
Tumor necrosis factor has been shown to induce cytostasis or cytoloysis in some but not all tumor cells. In order to investigate the early events which occur in TNF-treated tumor cells and their importance to the TNF antiproliferative response TNF-resistant cells (ME-180R) were clonally derived from a TNF-sensitive cervical carcinoma cell population (ME-180P). Both ME-180R and ME-180P cells were shown to bind TNF with equal affinity and express similar quantities of TNF receptors on their cell surface. Quantitation of EGF receptor expression by immunoblotting demonstrated a 3-fold greater expression in ME-180R cells when compared to TNF-sensitive ME-180P cells. In addition, the basal state of EGF receptor tyrosine kinase activity and phosphotyrosine content were significantly higher in TNF-resistant ME-180R cells. TNF treatment of ME-180P cells resulted in a 3-fold increase in the state of EGF receptor phosphorylation which was similar to the effects of EGF. TNF was unable to significantly alter the state of EGF receptor phosphorylation in TNF-resistant ME-180R cells. These results suggest that the intrinsic state of EGF receptor phosphorylation, its expression and its activation by TNF are important early events in the biological response to TNF.

D 113 TYROSINE PHOSPHORYLATION IN NK CELLS PROVIDES AN EARLY SIGNAL FOR THE ACTIVATION OF CYTOTOXIC FUNCTION. Kregg J. Einspahr*, Robert T. Abraham*+, Bryce A. Binstadt* and Paul J. Leibson*, Departments of *Immunology and +Pharmacology, Mayo Clinic and Foundation, Rochester, MN 55905
Recent evidence suggests that protein tyrosine phosphorylation transduces early activation signals and regulates cell-type specific functional responses in non-transformed cell types. In light of such evidence and because the molecular mechanisms of NK cell activation are incompletely understood, we undertook a systematic study of the role which protein tyrosine phosphorylation plays in the activation of cytolytic function in clonal $CD16^{+}/CD3^{-}$ human NK cells. We demonstrated that stimulation of the Fc receptor (CD16), rapidly induced the tyrosine phosphorylation of a number of NK cell proteins. These effects occur within 2 min and are maximal at 10 min following Fc receptor ligation. Fc receptor stimulation in intact cells increased the *in vitro* protein kinase activity present in anti-phosphotyrosine antibody immunoprecipitates from these cells. Furthermore, direct contact of NK cells with tumor cells susceptible to NK cell cytotoxic action induced a rapid (within 2 min) increase in the tyrosine phosphorylation of several intracellular proteins and these effects appear to be associated with the susceptibility of the tumor cell line to NK cell-mediated cytolysis. In addition, we demonstrate that the tyrosine kinase inhibitors, genistein and herbimycin A, are capable of blocking NK cytolytic function in a dose-dependent manner. Our results indicate that protein tyrosine phosphorylation may be an obligatory early proximal signal in activating the antibody-dependent and cell-mediated cytotoxic function of NK cells.

D 114 **PURIFICATION, CHARACTERIZATION AND SPECIFICITY OF A TKP FROM HL-60,** A.P. Ernould, G. Ferry, A. Genton, J.M. Barret, G. Atassi and J.A. Boutin, Institut de Recherches SERVIER- Division de Cancérologie Expérimentale - 11, rue des Moulineaux - 92150 Suresnes -FRANCE.

Phosphorylation of tyrosine residues is involved in growth signal transmission. In our attempt to find more specific anticancer compounds, we purified a tyrosine protein kinase from a leukemia cell line (HL60) by Triton X100 mild solubilization in presence of protease inhibitors.

Purification scheme is presented. Particular features of this TPK include poor stability, rather low molecular weight, quite hydrophobic quality and activation by ionic strength. Furthermore, the enzyme seems not to be either autophosphorylated or recognized by anti v-src antibodies. Extensive specificity studies were conducted by two original methods. The first one uses a conventional reverse phase and the second one is a HPLC hydrophilic interaction chromatography (HILIC mode). More than 200 peptides were studied including angiotensin- and minigastrin-derived structures. As expected, acidic residues are shown to play a major role in the substrate recognition by the enzyme.

Growth Factor Signal Transduction

D 115 MECHANISMS OF EGF-INDEPENDENCE IN RAT MAMMARY CARCINOMA CELLS.

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Rat mammary carcinoma (RMC) cell lines derived from serially transplantable mammary tumors are independent of epidermal growth factor (EGF) for long-term growth in serum-free medium. This phenotype is in stark contrast to that expressed by cells obtained from either normal mammary tissues or non-transplantable mammary tumors which express an absolute requirement for exogenous EGF for growth in serum-free culture. The present studies were aimed at examining the cellular mechanisms for the EGF-independence of the RMC cells. Examination of conditioned media obtained from EGF-independent cells indicated the presence of potent EGF-like bioactivity. Four of four EGF-independent cell lines examined secreted an activity into conditioned medium that substituted for EGF for the growth of the EGF-dependent cell line MCF-10. However, this activity appears not to be transforming growth factor (TGF)-alpha as both radioimmunoassays and enzyme linked immunoabsorbant assays of conditioned media obtained from EGF-independent cells were negative. In addition, radio-receptors assays indicated that this activity does not compete with EGF for binding the EGF-receptor. Western blot analysis of cell lysates obtained from EGF-independent cells probed with the phosphotyrosine antibody Py-20 revealed a prominent 180 to 190 kDa band that was distinct from the EGF-receptor band detected in lysates of EGF-dependent cells. Immunoprecipitation of EGF-receptors from EGF-independent cells using a rat EGF-receptor antiserum confirmed the differential migration of the rat EGF-receptor and the tyrosine phosphorylated band detected in Western blots. Taken together, these results suggest that an autocrine loop mediates independence of exogenous EGF in RMC cells but that this autocrine loop operates outside of the EGF/TGF-alpha-EGF-receptor system. Experiments currently in progress are aimed at determining if the the 185 kDa band detected in phospho-tyrosine western blots is the c-erbB-2 protein. Supported by grant CA 40064 from the NCI.

D 116 DECLINE OF RECEPTOR-MEDIATED SECOND MESSENGER PATHWAYS IN IMR90 DIPLOID FIBROBLASTS AT HIGH POPULATION DOUBLINGS,

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The human diploid fibroblast strain IMR90 undergoes cell senescence accompanied by a loss of responsiveness to growth factors at high population doubling levels (PDL). Hydrolysis of PIP_2 in response to serum stimulation of IMR90 was measured in an *in vitro* assay. The phospholipase C (PLC) activity in PDL 21-23 cells (31-34% of lifespan expended) was stimulated 5-fold in response to 10% serum compared to 3-fold in PDL 57-59 cells (84-87% of lifespan expended). In immunoaffinity purified tyrosine phosphorylated proteins from serum-stimulated cells, PLC activity was greater in low versus high PDL cells. Ten percent fetal bovine serum stimulated phosphorylation of 185, 91,, 85, and 74 KD proteins as analyzed by *in vitro* kinase assay of antiphosphotyrosine immunoprecipitates. Comparison of the stimulated, phosphorylated proteins indicated the presence of a 67 kD protein only in low PDL cells. Phosphatidylinositol 3-kinase (PtdIns 3-kinase), an enzyme of putative molecular weight 85kD, is associated with several tyrosine kinase oncogenes in transformed cells and can also be activated by several growth factors in normal cells. Serum stimulation of PtdIns 3-kinase in IMR90 cells was diminished at high PDL. The product of this enzymatic reaction was shown by HPLC to migrate at the position of PtdIns 3-phosphate. These studies demonstrate diminution of two receptor-coupled pathways believed to have important roles in signal transduction and growth stimulation in an *in vitro* model of cellular senescence.

D 117 MULTIPLE TRANCRIPTS OF THE C-MET ONCOGENE,

Silvia Giordano, Carola Ponzetto, Lucia Gandino, Giovanni Gaudino, Enzo Medico, M. Flavia Di Renzo and Paolo M. Comoglio, Department of Biomedical Sciences & Oncology - University of Torino - Italy

The proto-oncogene *C-MET* encodes a transmembrane protein with structural features of a tyrosine kinase receptor. The *C-MET* protein is a heterodimer of two disulphide linked chains: α of 50 kd and β of 145 kd (Giordano et al., Nature, 339, 155-156, 1989). The C-MET product is first synthesized as a single chain precursor of 170kd which undergoes a rearrangement of disulphide bonds before being cleaved (Giordano et al., Oncogene, 4, 1383-1388, 1989). The tyrosine kinase activity of the C-MET protein is negatively modulated by protein kinase C. Activation of PKC increases C-MET serine phosphorylation and decreases C-MET tyrosine phosphorylation (Gandino et al., Oncogene, 5, 721-725, 1990). We studied a gastric tumor cell line where the C-MET protein is constitutively phosphorylated on tyrosine and the *C-MET* gene is amplified and overexpressed. Northern analysis reveals the presence of four major mRNA transcripts of 9.0, 7.0, 5.2 and 3.4 kilobases. A cDNA library was constructed in λGT11 vector with poly(A) RNA, using random hexanucleotides as primers. A series of clones spanning the entire *C-MET* gene were isolated and sequenced. cDNA clones and PCR probes corresponding to different portions of the sequence were used to characterize the multiple mRNA transcripts. Probes encompassing the first 1300 nucleotides of the 5' portion of the coding sequence do not hybridize with the 7 and 5 kb transcripts. These transcripts could potentially code for proteins truncated in the extracellular domain or possessing entirely different N-termini. Since the 7 kb mRNA is common to a number of cell lines, the presence of mRNAs derived from the use of alternative 5' exons seems to be a common feature of the transcription of the normal *C-MET* gene. We sequenced the cloned cDNAs and found three main differences respect to the published sequence (Park et al., PNAS, 84, 6379-6383, 1987): (1) the lack of 54 nucleotides corresponding to a stretch of 18aa located in the extracellular domain of the receptor (2) the substitution of the codon specifying alanine 1209 with one coding for glycine (3) the insertion of a 14^0 mer in the 3'-untranslated region. We obtained cDNAs identical to that just described from a number of control cell lines; these results suggest that the present C-MET cDNA presumably reflects the sequence of the most abundant transcript in several human tissues.

D 118 HEAVEY CHAIN CLATHRIN IS A SUBSTRATE FOR INSULIN RECEPTOR TYROSINE KINASE BUT PLASMA MEMBRANE PP180 IS NOT PHOSPHORYLATED CLATHRIN. H. Joseph Goren, Marilyn J. Mooibroek and Donna Boland. Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada T2N 4N1
Much evidence supports a role for receptor tyrosine kinase activity for insulin receptor (IR) endocytosis. One IR substrate, *pp180, associated with plasma membranes, with cytoskeletal proteins and found in the cytoplasm, migrates like clathrin heavy* chain in dodecyl sulphate gel electrophoresis (SDS-PAGE). Since clathrin heavy chain is a component of coated pits, and coated pits function in IR endocytosis, we have examined the ability for clathrin heavy chain to function as an IR substrate, and whether pp180, the plasma membrane-associated IR substrate is phosphorylated clathrin heavy chain. Bovine brain triskelion was added to wheat germ agglutinin (WGA) purified human placenta plasma membranes in the presence of ^{32}P-ATP and $MnCl_2$. Antiphosphotyrosine-immunoprecipitated proteins were analyzed with SDS-PAGE. The yield of ^{32}P-labeled 180 kDa protein was dependent: on insulin concentrations, on reaction time, on insulin receptor concentration, and on triskelion concentration. Similarly, human placenta coated vesicles yielded a ^{32}P-labeled 180 kDa protein in the presence of WGA-purified placenta membranes, insulin, and ^{32}P-ATP. Further, monoclonal anti-heavy chain clathrin antibody immunoprecipitated triskelion ^{32}P-labeled 180 kDa protein. Taken together, these results indicate that IR catalyzes clathrin heavy chain phosphorylation. Placenta plasma membrane pp180, however, was not immunoprecipiatated with anti heavy chain clathrin, nor was its yield lowered in WGA-purified membranes pretreated with anti-heavy chain clathrin, suggesting that pp180 is likely not phosphorylated heavy chain clathrin.

D 119 PHOSPHOINOSITIDE 3-KINASE AND PHOSPHATYDILINOSITOL(3)MONO-PHOSPHATE 4-KINASE IN PP190^{C-MET} TRANSFORMED HUMAN GASTRIC TUMOR CELLS. Andrea Graziani[1,2], Lewis C. Cantley[2], Paolo Comoglio[1] [1]Department of Biomedical Sciences and Oncology, University of Torino Medical School, Cs.so M. D'Azeglio 52, 10126 Torino, Italy [2]Department of Physiology, Tufts University Medical School , 136 Harrison Ave., Boston, MA 02111.
GTL16, a human gastric tumor cell line, contains high levels of pp190^{c-met}, a tyrosine kinase receptor-like protein. Phosphoinositide 3-kinase, a promiscous enzyme that phosphorylates the D-3 position of PtdIns, PtdIns(4)P and PtdIns(4,5)P_2, appears to associate with all the receptor type tyrosine kinases that have been investigated so far. We demonstrated that in GTL16 cells the phosphoinositide 3-kinase is immunoprecipitated by both anti-phosphotyrosine and anti-pp190^{c-met} antibodies. We also observed detectable levels of the phopshoinositide 3-kinase products, PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, in [^{3}H]-inositol labelled GTL 16 cells. Recent evidences indicate that in platelets and human erythrocytes, PtdIns(3,4)P_2 can also be synthesyzed via D-4 phopshorylation of PtdIns(3)P by a novel PtdIns(3)P 4-kinase activity. We observed a similar PtdIns(3)P 4-kinase activity in GTL16 lysates. The discovery of such enzymatic activity leads to speculation that PtdIns(3,4)P_2 can be synthesyzed via two alternative pathways in cells expressing high level of pp190^{c-met}.

D 120 EXPRESSION OF ACTIVE NORMAL AND ONCOGENIC FORMS OF THE NEU TYROSINE KINASE IN INSECT CELLS. Pamela M. Guy, John G. Koland, Kermit L. Carraway and Richard A. Cerione, Department of Biochemistry, Cell and Molecular Biology, Cornell University, Ithaca, NY 14853.
We have expressed full-length and truncated forms of the normal and oncogenic forms of the neu tyrosine kinase in insect cells using a baculovirus expression system. We are using these proteins to address three questions regarding the role of the neu tyrosine kinase in signal transduction. First, we are trying to quantitate the difference in the intrinsic kinase activities of the normal and oncogenic neu, using full-length and erb-B-like forms of the protein. Second, we are interested in determining whether or not the neu tyrosine kinase might serve in a signaling pathway of the EGF receptor by interacting synergistically with the EGF receptor tyrosine kinase. These studies are being performed with a soluble intracellular domain of the neu tyrosine kinase, and the EGF receptor from plasma membranes of A431 cells. Finally, we are interested in comparing the phosphorylation of different candidate phosphosubstrates by the full-length and truncated forms of the normal and oncogenic neu tyrosine kinases.

D 121 IDENTIFICATION AND CHARACTERIZATION OF THE PDGF RECEPTORS IN CELLS DERIVED FROM HUMAN BENIGN PROSTATIC HYPERPLASIA,

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Prostatic hyperplasia (BPH) is characterized by chronic inflammation and stromal fibrosis along with abnormal stromal proliferation, and the accumulation of extracellular matrix. Although the prostate is dependent upon androgens for its growth and normal physiologic function, it has recently been suggested that androgens might be acting indirectly by regulating the expression of growth factors and growth factor receptors. The focus of these studies was to elucidate the potential role of PDGF in the induction of cellular hyperplasia. Surgical specimens of human prostatic tissue displayed the proliferation of glands and stroma typical of prostatic hyperplasia with some samples exhibiting chronic inflammation. The tissues were minced and chemically dissociated and the cells placed in culture. Using selective media, cultures of both epithelia and stromal cell were established. In situ hybridization was performed using [^{35}S] riboprobes specific for the mRNA which codes for either the $PDGF_A$ or $PDGF_B$ receptor subunits. Messenger RNA for the $PDGF_B$ receptor was found in most of the cellular elements with a slightly more robust hybridization signal over the epithelial cells. Hybridization to the $PDGF_A$ receptor mRNA was limited. Cultures of prostate cells were also stained with antisera for the $PDGF_{BB}$ receptor using the peroxidasse-antiperoxidase technique. Staining was found in both epithelial cells and fibroblasts. Using receptor binding techniques, BPH-derived prostate cells were found to have high affinity PDGF receptors with K_D values which ranged from 0.186 to 9.12 nM with 87,000 to 1,410,000 sites per cell. The prostatitis associated with BPH and the resulting inflammation could lead to the release of PDGF which is known to function as a chemo-attractant for fibroblasts and smooth muscle cells and to promote their proliferation. The presence of functional PDGF receptors in human prostatic tissue lends support to the hypothesis that PDGF may play a key role in the etiology of BPH.

D 122 Control of nuclear localization and activation of transformation in the c-*abl* gene product: the role of SH3, SH2, kinase activity, and phosphorylation.

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Mutational analysis of c-*abl* previously suggested the presence of a nuclear localization signal. By use of an epitope-tagged c-*abl*, we map a second C-terminal nuclear localization signal. Mutations in SH3 appear to coordinately activate transforming ability and relocalization to the cytoplasm of a normally nuclear c-*abl*. We demonstrate that relocalization is dependent on N-terminal mutations, and not dependent on the transformed state of the cells, suggesting a mechanism for regulation of nuclear localization. Relocalization from nucleus to cytoplasm appears to be independent of kinase activity and autophosphorylation state.

We have developed a filter binding assay for the ability of a bacterially expressed SH2 domain to bind specifically to phosphotyrosinated proteins. We are currently studying the effect of mutations in conserved residues in SH2 on their ability to (1) activate a normal c-*abl;* (2) abrogate the transforming ability of an SH3 mutated, activated abl; (3) restore the nuclear localization of these transformation defective mutants; and, (4) reduce the ability of the bacterially expresed SH2 to bind phosphotyrosinated proteins.

D 123 MUTATIONS IN THE JUXTAMEMBRANE REGION OF THE INSULIN RECEPTOR IMPAIR ACTIVATION OF PHOSPHATIDYLINOSITOL 3-KINASE BY INSULIN,

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Insulin activates Phosphatidylinositol 3-kinase (PtdIns 3-kinase). To investigate further the role of PtdIns 3-kinase activity in insulin action, we examined its modulation in CHO cells expressing an insulin receptor mutant (CHO/IR_{F960}) which is defective in transmitting the insulin signal but undergoes autophosphorylation normally. PtdIns 3-kinase activity in anti-phosphotyrosine [anti-tyr(P)] immunoprecipitates from insulin-stimulated CHO/IR_{F960} cells is 20-40% of that in CHO/IR cells; IGF-1 stimulated PtdIns 3-kinase activity in anti-tyr(P) immunoprecipitates is similar in the two cell types. Insulin failed to stimulate phosphate-incorporation into phosphatidylinositol-3,4-bisphosphate [$PtdIns(3,4)P_2$] in CHO/IR_{F960} cells whereas it caused a 12 fold increase in CHO/IR cells. In contrast phosphate-incorporation into phosphatidylinositol trisphosphate [$PtdInsP_3$], was similar in the two cell types. The amount of the 85 kDa subunit of the PtdIns 3-kinase in anti-Tyr(P) immunoprecipitates of insulin stimulated CHO/F960 cells is also diminished compared to that in CHO/IR cells. These results suggest that the juxtamembrane region of the insulin receptor is important for activation of PtdIns 3-kinase. They also suggest that the formation of $PtdIns(3,4)P_2$ and $PtdInsP_3$ may be differentially regulated in the intact cell and that production of the former species more closely parallels the biological action(s) of insulin.

D 124 A NEW METHOD FOR CHARACTERIZING BINDING OF PHOSPHORYLATED PLATELET-DERIVED GROWTH FACTOR RECEPTORS TO AN 85KD SIGNALLING MOLECULE, W. Michael Kavanaugh, Jaime A. Escobedo, Lewis T. Williams, Cardiovascular Research Institute, University of California, San Francisco, CA. 94143
Physical association of cytoplasmic signalling molecules with the activated PDGF receptor is thought to be an important event in signal transduction. We have used a modification of the ligand blotting technique to examine binding of the PDGF receptor to one such molecule, p85/PI Kinase. 32-P-labeled, baculovirus-expressed PDGF receptor was used as a probe to blot SDS PAGE- fractionated 3T3 cell lysates that had been transferred to nitrocellulose. The labeled, phosphorylated PDGF receptor bound directly to an 85Kd protein in quiescent 3T3 lysates. This binding was specifically blocked by a short, phosphotyrosine-containing peptide derived from the kinase insert domain of the PDGF receptor. No binding to p85 was observed when PDGF receptor was used to probe lysates from PDGF-stimulated cells. This suggests that upon PDGF stimulation, p85 is degraded, or modified so that it no longer binds to the receptor. Treatment of PDGF-stimulated lysates with phosphatase restores p85 binding to the receptor probe. We conclude: 1) p85 can bind directly to phosphorylated PDGF receptors in the absence of other proteins; 2) this binding is mediated by a short receptor sequence containing phosphotyrosine; 3) p85 from PDGF-stimulated cells is not available for binding to the PDGF receptor because of a phosphorylation event which lowers p85 affinity for the receptor. Characterization of this phosphorylation event, which may be important in control of signalling by p85/PI Kinase, is currently underway.

D 125 BINDING OF TYROSINE PHOSPHORYLATED PROTEINS TO THE *SRC* HOMOLOGY DOMAINS OF GAP, Anne Koch, Deborah Anderson, Michael Moran, Christine Ellis, Luc Marengere and Tony Pawson, Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada M5G 1X5
The SH2 domain is a noncatalytic region of approximately 100 amino acids conserved between cytoplasmic protein-tyrosine kinases. Genetic and biochemical data indicate that the SH2 domains of the Fps and Src tyrosine kinases interact with the catalytic domain to regulate kinase activity, and is also required for the accumulation of specific tyrosine phosphorylated proteins in *fps*- or *src*-transformed cells. SH2 domains have also been identified in several catalytically unrelated proteins, including phospholipase C γ, GTPase activating protein (GAP) and the v-*crk* oncoprotein. Under appropriate circumstances, these SH2-containing proteins can form complexes with one or more tyrosine phosphorylated proteins. We have shown that several of these associations can be reconstituted in vitro using bacterial fusion proteins containing the SH2 regions of these signalling or transforming proteins. For example, a GAP SH2 domain is sufficient for the binding of activated EGF- or PDGF-receptors. These results suggest that the interactions of cytoplasmic signalling proteins may be regulated by their SH2 domains. A more detailed analysis of the binding specificities of the GAP SH2 regions will be presented.

D 126 CD45 EXPRESSION IS ESSENTIAL FOR T CELL ANTIGEN RECEPTOR AND CD2 MEDIATED TYROSINE KINASE ACTIVATION AND INTERLEUKIN 2 PRODUCTION, Gary A. Koretzky, Joel Picus, Terrie Schultz, and Arthur Weiss, Department of Medicine, Cancer Research Institute, and Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA 94143
CD45, an hematopoetic cell specific surface protein tyrosine phosphatase, has recently been shown to be important in T cell activation. We have taken a genetic approach to address the role of this phosphatase in regulating signal transduction via the T cell antigen receptor (TCR) by selecting for CD45-deficient mutants of two human T cell leukemic lines. The TCR in our CD45-deficient cells is uncoupled from both the phosphatidlyinositol (PI) and tyrosine kinase (PTK) second messengers pathways. While the CD45-deficient cells are capable of producing interleukin 2 when treated with pharmacologic agents, TCR stimulation does not result in production of this lymphokine. A similar signalling defect is seen in the CD45-deficient cells when activation via the accessory molecule, CD2, is attempted. However, signal transduction is not globally inhibited in the CD45-deficient cells because activation via another surface antigen, CD28, or via a transfected heterologous receptor, the human muscarinic receptor, appears to be unimpaired. Thus, CD45, perhaps by controlling a common regulatory process, appears to be essential to specifically link the TCR to both the PTK and PI signal transduction machinery in human T lymphocytes.

D 127 PDGF-INDUCED PROLIFERATION OF CELLS DERIVED FROM HUMAN BENIGN PROSTATIC HYPERPLASIA. Tracey D. Kriauciunas[1], Kenneth S. Hirsch[1], Jeffery A. Jones[2], John R. Regan[2], John N. Eble[3], Julie F. Falcone[1], and Chris J. Vlahos[1], [1]Lilly Research Laboratories, Indianapolis IN 46285, [2]Department of Urology, and [3]Department of Pathology, Indiana University Medical Center, Indianapolis, IN 46202.
Benign prostatic hyperplasia (BPH) is a disease characterized by abnormal proliferation of the cells comprising the prostate. Previous findings have shown that cultured human prostate cells contain mRNA for the PDGF$_B$ receptor, as well as high affinity PDGF receptors (K_d=0.186-9.12 nM with 87,000-1,410,000 sites per cell). The experiments presented below demonstrate that PDGF receptor is activated in response to the growth factor and that mitogenesis is induced. Treatment of cultured prostate cells with PDGF$_{BB}$ activates the signal transduction pathway of the PDGF receptor as evidenced by the presence of several phosphoproteins in antiphosphotyrosine immunoprecipitates of the stimulated cells, including autophosphorylation of the PDGF receptor. Phosphatidylinositol kinase activity is also increased in cells stimulated with PDGF. Depending on the sample, the addition of PDGF to the media caused a dose-dependent increase in [^{3}H]-thymidine incorporation with a peak 2-20 fold increase occuring at 10-25 ng PDGF/ml. A dose-dependent increase in cell number was observed with exposure to PDGF with a maximal response of 2-3 fold increase occuring at 25 ng PDGF/ml. These studies demonstrate that PDGF initiates the signal transduction cascade in BPH-derived cells and subsequently leads to mitogenesis in vitro.

D 128 EXPRESSION OF P210*bcr/abl* IN 32D Cl3(G) CELLS RESULTS IN IL-3 INDEPENDENT GROWTH AND BLOCKS PGE$_1$ MEDIATED INHIBITION

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The chronic myelogenous leukemia (CML) associated *bcr/abl* gene was introduced into the IL-3 dependent 32D Cl3(G) cell line using retroviral mediated gene transfer. IL-3 dependence was abrogated in p210*bcr/abl* expressing cells (determined by invitro kinase assay and Westernblotting with anti-phosphotyropsine antibody), by a non-autocrine mechanism. In serum free conditions, the growth of transformed cells, in the absence of IL-3, matched that of IL-3 stimulated parental cells infected with vector not containing *bcr/abl*. In contrast, transformed cells proliferated more rapidly than control cells in the presence of serum, (an effect not due to IGF-I and IGF-II). Expression of p210*bcr/abl* was associated with constitutive phosphorylation of proteins observed only with IL-3 stimulation in parental cells. The proliferation of 32D Cl3(G) cells was inhibited by cAMP analogues, forskolin and PGE$_1$, but not by pertussis toxin. In contrast, of 13 transformed independent clones examined, all were resistant to PGE$_1$, 5/13 were also relatively resistant to forskolin and cAMP analogue mediated inhibition, while the the other 8/13 clones remained sensitive to forskolin and cAMP analogues. These studies suggest that the proliferation of 32D Cl3(G) cells transformed by p210*bcr/abl* is a result of constitutive activation of components involved in IL-3 signal transduction and, by simultaneous blockage of a negative regulatory pathway of growth in myeloid cells.

D 129 STRUCTURE AND FUNCTION RELATIONSHIPS IN THE CSF-1 RECEPTOR, Angel Lee and Arthur Nienhuis, Clinical Hematology Branch, NIH, Bethesda, Md 20892.
The receptor for colony-stimulating factor 1 (CSF-1) is a member of the receptor tyrosine kinase (RTK) family, and is found on the surfaces of cells of the monocyte-macrophage lineage and on placental trophoblasts. One of the intracellular substrates of the CSF-1 receptor (CSF-1R) is a novel phosphatidyl inositol (PI) 3-kinase which may play a key role in mitogenesis and proliferation. The activated ligand-receptor complex is usually downregulated by internalization and lysosomal degradation. We wish to examine (i) whether an activated CSF-1R kinase contains all the necessary information for signal transduction and (ii) the nature of the determinants of down-regulation. Chimeric receptors between the external domain of glycophorin A (GpA) and the cytoplasmic domain of CSF-1R were constructed. GpA is an erythrocyte structural membrane protein with no known role in signal transduction and whose external domain bears no relation to RTKs. To examine the role of the transmembrane domain (TM) in kinase activation, two types of chimeric receptors were made, A (TM from GpA) and B (TM from CSF-1R). The plasmids containing the hybrid cDNAs were electroporated into 32D cells, a murine IL-3-dependent myeloid cell line. We find that polyclonal anti-GpA antibodies were able to activate the kinase domain of the chimeric receptors, thus supporting an oligomerization mechanism for ligand activation. Kinase activation was accompanied by mitogenesis that could be specifically blocked by exogenous GpA. A consistently high basal level of mitogenesis was found for the A type of receptors. Despite a foreign external/TM domain, both types of chimeric receptors were able to associate with PI 3-kinase, indicating that activation of the receptor kinase domain is the major determinant for coupling. The chimeric receptors were internalized in the presence of anti-GpA antibodies but were not degraded; even molecules that had undergone tyrosine phosphorylation were stable. Our results suggest that kinase activation is a necessary but insufficient signal for receptor degradation and that additional signals must lie in the external ligand-binding domain that target for lysosomal degradation.

D 130 MEMBRANE-ANCHORED FORM OF V-SIS/PDGF-B INDUCES MITOGENESIS BUT NOT PDGF RECEPTOR AUTOPHOSPHORYLATION, Bruce A. Lee[1] and Daniel J. Donoghue[2], Departments of Biology[1] and Chemistry[2], and Center for Molecular Genetics, University of California San Diego, La Jolla, CA 92093.

Autophosphorylation of the PDGF receptor (PDGFR) is indicative of receptor activation and is thought to be important in mitogenic signal transduction. Whether or not autophosphorylation is absolutely required for a mitogenic or transforming response is not clear. Forms of the v-*sis* protein which are anchored to the cell membrane via the transmembrane domain of the vesicular stomatitis virus G protein have been previously described (Hannink and Donoghue. 1986. *J. Cell Biol.* 103:2311). Several of these fusion proteins were shown to interact productively with the PDGFR based on their ability to transform NIH 3T3 cells. Further characterization of one of these membrane-anchored v-*sis* proteins, designated v-sis^{239}-G will be presented. The gene encoding v-sis^{239}-G was placed under control of the *Drosophila melanogaster hsp70* promoter and inducible synthesis of this protein was shown to lead to a mitogenic response in NIH 3T3 cells. Unexpectedly, v-sis^{239}-G did not induce detectable receptor autophosphorylation, in contrast to a similarly expressed secreted form of the v-*sis* protein. Thus, it appears that a PDGFR-mediated mitogenic response can be dissociated from detectable receptor autophosphorylation. Furthermore, induced synthesis of v-sis^{239}-G was shown to lead to c-*fos* expression even in the absence of detectable autophosphorylation. Interestingly, a non-mitogenic membrane-anchored form of the v-*sis* protein, designated v-sis^{239}-G^{338}, also induced c-*fos* without receptor autophosphorylation. These results suggest the possibility of a PDGFR-mediated autophosphorylation-independent signal transduction pathway.

D 131 EXPERIMENTAL MANIPULATION OF P56^{lck} KINASE ACTIVITY AND ITS EFFECT ON THYMOCYTE DEVELOPMENT, Steven D. Levin, Kristin M. Abraham, Katherine A. Forbush, and Roger M. Perlmutter, Departments of Biochemistry and Immunology and the Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195.

The product of the *lck* proto-oncogene (p56^{lck}) is a membrane-associated protein tyrosine kinase preferentially expressed in T lymphocytes. It is normally found in association with the CD4 and CD8 co-receptors and thus is circumstantially implicated in signal transduction from the lymphocyte surface. To study the nature of this signal, we sought to determine the effects of increased p56^{lck} kinase activity during thymocyte development. By over-expressing the wild-type (LGY) or a constitutively activated form of the kinase (LGF) in thymocytes, we were able to perturb thymic development in a predictable fashion dependent on *lck* kinase activity. Animals expressing high levels of the transgenes developed thymic tumors while animals expressing lower levels showed a loss of surface CD3, exhibited retarded thymic development, and produced mature T cells which were refractory to stimulation. The severity of these defects were correlated with transgene expression levels. To further dissect these abnormalities transgenic animals were generated over-expressing an activated form of p56^{lck} containing mutations in two amino-terminal cysteine residues known to be essential for interaction of the kinase with CD4 and CD8 (LGCAF). Analysis of these animals suggests that T cell signaling and development are dependent upon the association of p56^{lck} with CD4 and CD8.

D 132 REGULATION OF GAP BY TYROSINE PHOSPHORYLATION, Xingquan Liu and Tony Pawson, Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, 600 University Avenue,Toronto, Ontario, M5G 1X5 Canada

GAP (GTPase activating protein) is phosphorylated on tyrosine in cells transformed by oncogenic tyrosine kinases or following growth factor stimulation in cells over-expressing the cognate growth factor receptor (Ellis, C., Moran, M., McCormick, F., and Pawson, T. 1990, Nature, 343, 377-381). We are interested in the regulation of GAP through tyrosine phosphorylation by these receptor and cytoplasmic tyrosine kinases. Purified GAP from a baculovirus expression system was phosphorylated *in vitro* by the EGF-receptor kinase domain resulting in phosphorylation on a single tyrosine residue. Upon digestion by trypsin and analysis on a 2-D TLC plate, we have shown that this phosphorylation site corresponds to the single major tyrosine residue which is phosphorylated *in vivo* by both EGF-receptor and p60$^{v\text{-}src}$. Phosphopeptide mapping and mutagenesis are being employed to determine the tyrosine residue on GAP that is modified. The possible effect of this tyrosine phosphorylation on GAP activity and the association of GAP with other signalling molecules are also being studied. These results will be presented.

D 133 METHODS OF INHIBITING GROWTH FACTOR RECEPTOR DIMERISATION, Fiona J. Lofts, Helen C. Hurst, Michael J.E. Sternberg and William J. Gullick, ICRF Oncology Group, Hammersmith Hospital, London W12 0HS, U.K.
The *neu* proto-oncogene encodes a polypeptide transmembrane molecule bearing considerable homology to the epidermal growth factor receptor (EGFr). The activated oncogenic counterpart of *neu* has a single point mutation within the hydrophobic transmembrane domain resulting in a glutamic acid for valine substitution. This activated molecule has been shown to have a constitutively more active tyrosine kinase and forms dimers more readily than the proto-oncogene product when expressed in mouse fibroblasts. It has been proposed that the dimerisation of tyrosine kinase growth factor receptor molecules is necessary for kinase activity and propagation of mitogenic signal, and that the point mutation in *neu* may stabilise the dimeric form. In an attempt to inhibit this process using the *neu* protein as a model, we have developed two approaches. The first was to design and synthesise 18 amino acid amphipathic peptides based on the primary structure of *neu* transmembrane sequence which were both soluble in aqueous solution and cell membranes and yet still form an alpha helix within the membrane and thus interact with the whole protein to form an inactive complex. The second approach was to express a truncated *neu* protein in mouse fibroblasts transformed by expression of activated *neu* protein. We present data on the development of both peptides and transfected cell clones and their effect on the transformed phenotype.

D 134 *IN-VITRO* SUBSTRATE SPECIFICITY OF HYBRID TYROSINE PROTEIN KINASES, GENERATED BETWEEN C-SRC AND V-ABL BY RECOMBINATION WITHIN CONSERVED CATALYTIC DOMAIN MOTIFS.
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Recent findings have strongly suggested that a major determinant of the biological behaviour of protein tyrosine kinases (TPK) is catalytic specificity, encoded within their conserved kinase domains (1-3). Alignment of the kinase domains of TPKs reveals a pattern of conserved regions which are postulated to play important roles in catalytic function (4). The v-abl and v-src oncogenes, although highly related in amino acid sequence, display distinct biological properties, presumably due to differences in substrate specificity (1). To help understand the structural basis of catalytic specificity, we have constructed a number of hybrid enzymes by recombination within conserved catalytic domain motifs of v-abl and c-src. The results of substrate specificity studies *in vitro* using hybrid and parent enzymes will be reported.

1.Mathey-Prevot B. and Baltimore D. (1988) MCB 8, 234-240
2.Privalsky M.L. (1987) J. Viol. 61, 1938-1948.
3.Di Fiore et al., (1990) Science 248, 79-83
4.Hanks et al., (1988) Science 241, 42-52

D 135 GROWTH RESPONSE OF SQUAMOUS CELL CARCINOMA SPHEROIDS TO EGF: IMPORTANCE OF PROTEIN TYROSINE PHOSPHATASE. JN Mansbridge, AM Knapp, TT Kwok, R Knüchel, RM Sutherland. Cancer Biology, SRI International, Menlo Park, CA 94025.

The growth of A431 and CaSki cells cultured as spheroids shows a dependence on EGF which is not seen in monolayer cultures. In seeking a mechanism for this finding, we have shown, by Scatchard analysis, the frequency of surface EGF receptors is reduced in spheroids 3-7 fold. In addition, we have found an increased rate of dephosphorylation of the EGF receptor in high density cultures and spheroids.

Immunohistochemical staining of cultures of varying density by conventional techniques showed an inverse relationship between EGF stimulated tyrosine phosphorylation and cell density. This effect was lost when phosphatase inhibitors, particularly 1 mM vanadate and zinc, were added. In spheroids, tyrosine phosphorylation could only be demonstrated in the presence of phosphatase inhibitors. When treated for 10 min with 500 ng/mL EGF, a concentration which was shown to penetrate throughout the spheroid, staining was confined to the outermost layer of cells.

The detection of protein tyrosine phosphate on immunoblots depended critically on the presence of phosphatase inhibitors during extraction, particularly in the cases of dense cultures and spheroids. The requirement for special precautions to eliminate phosphatase activity in dense cultures and spheroids, which were not necessary in sparse cultures, led us to propose an increase in cellular tyrosine phosphatase activity with cell density. The response of the cells to the spheroid cellular microenvironment by a reduced number of receptors and increased phosphatase activity results in an overall reduction in EGF receptor phosphorylation. This provides a partial explanation for the dependence of spheroid growth on supplemental EGF.

D 136 *trk*B EXPRESSES BOTH A RECEPTOR PROTEIN-TYROSINE KINASE AND TRUNCATED RECEPTORS IN THE NERVOUS SYSTEM, David S. Middlemas and Tony Hunter, Molecular Biology and Virology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, CA 92186-5800

In order to find novel genes which may play a role in neuronal signal transduction, we screened a rat cerebellar cDNA library with degenerate oligonucleotide probes which correspond to receptor protein-tyrosine kinases (PTKs). We identified a cDNA for a putative PTK, *trk*B, which shares sequence homology in both the extracellular region and kinase domain with the human *trk* proto-oncogene. The sequence of a cDNA containing the entire coding region indicates *trk*B is likely to encode a ligand-regulated receptor. Analysis of RNA extracted from rat tissues shows that *trk*B is expressed predominantly in the brain and there are multiple mRNAs, ranging from 0.7 to 9 kb. To resolve whether these mRNAs encode different proteins, Northern analyses of cerebral RNA using *trk*B probes which hybridized to either the extracellular or kinase domains were carried out. This analysis indicated there are mRNAs encoding C-terminally truncated *trk*B receptors. Consequently, we searched for and identified additional cDNAs encoding two distinct C-terminally truncated receptors which lack the kinase domain. These cDNAs encode the complete extracellular region and transmembrane domain, but have different short cytoplasmic tails of about 20 amino acids. We are currently raising antibodies specific for the different forms to investigate the enzymatic properties and the regional and cellular localization of these proteins in the brain.

D 137 NERVE GROWTH FACTOR STIMULATES PROTEIN TYROSINE PHOSPHORYLATION IN PC-12 PHEOCHROMOCYTOMA CELLS, Tadayo Miyasaka, David Sternberg*, Peter Sherline*, Junko Miyasaka and Alan R. Saltiel, Parke-Davis, 2800 Plymouth Road, Ann Arbor, MI 48106 and *Laboratory of Molecular Oncology, Rockefeller University, 1230 York Avenue, New York, NY 10021

PC-12 cells contain a growth factor-sensitive protein kinase that phosphorylates microtubule associated protein 2 (MAP-2). Two forms of this MAP kinase (I and II) can be resolved on phenyl superose. NGF and EGF stimulate the activity of both isoforms of the kinase. NGF and EGF also stimulate the phosphorylation on tyrosine of two proteins (pp42 and pp40), identified by immunoblotting of SDS gels with anti-phosphotyrosine antibodies. The kinetics and dose dependence of MAP kinase activation and tyrosine phosphorylation of pp42/40 by the growth factors are identical. Interestingly, the protein kinase inhibitors, staurosporine and K-252A selectively inhibit both the MAP kinase activation and the tyrosine phosphorylation of pp42 and pp40 by NGF, but are ineffective in attenuating the effect of EGF. However, comparison of the chromatographic properties of the growth factor-sensitive MAP kinase with pp42 and pp40 revealed that they were not identical. Purified MAP kinase II had no detectable phosphotyrosine. MAP kinase I did not coelute with pp40, although there was some overlap. Moreover, studies with okadaic acid and alkaline phosphatase reveal that serine phosphorylation is required for activation of both MAP kinase isoforms, although the role of tyrosine phosphorylation remains unclear. Thus, although NGF stimulates the rapid phosphorylation on tyrosine of proteins with characteristics similar to MAP kinase, it appears that these proteins are not identical to the major growth factor-sensitive isoforms of MAP kinase.

D 138 PHOSPHORYLATION OF $p34^{cdc2}$ ON TYROSINE WHEN CO-PRODUCED WITH THE PRODUCT OF $WEE1^{+}$ IN INSECT CELLS, L. L. Parker, S. Atherton-Fessler, and H. Piwnica-Worms, Tufts Medical School, Boston, MA 02111.

To study the $wee1^{+}$ gene product and its interactions with $p34^{cdc2}$, we have over-produced both proteins in insect cells using a baculovirus expression system. $p34^{cdc2}$ migrated as a single species by SDS-PAGE with an apparent molecular weight of 34Kd. The $wee1^{+}$ gene product ($p107^{wee1}$) was resolved into multiple species ranging in size from 107Kd to 110Kd. Both proteins were phosphorylated in insect cells; $p34^{cdc2}$ predominantly on threonine residues, and $p107^{wee1}$ primarily on serine residues. In immune complex kinase assays carried out in vitro with $p107^{wee1}$specific anti-serum, $p107^{wee1}$was phosphorylated on serine and threonine residues, and unexpectantly also on tyrosine residues. In contrast, a kinase-deficient mutant of $p107^{wee1}$was not phosphorylated under these conditions. Co-expression of $p34^{cdc2}$ and $p107^{wee1}$resulted in a population of $p34^{cdc2}$ with a retarded electrophoretic mobility. This population of $p34^{cdc2}$ was phosphorylated on tyrosine and not detectably on threonine residues. A mutant of $p34^{cdc2}$ (Tyr15-Phe) did not display an altered electrophoretic mobility when co-expressed with p107wee1, nor was it detectably phosphorylated on tyrosine residues. These results suggest that $p107^{wee1}$functions to regulate $p34^{cdc2}$, either directly or indirectly, by tyrosine phosphorylation.

D 139 Novel tyrosine kinases expressed in the K562 human leukemia cells

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Tyrosine phosphorylation is important in the transmission of growth and differentiation signals; known tyrosine kinases include several oncoproteins and growth factor receptors. Interestingly, some differentiated cell types, such as erythrocytes and platelets contain high amounts of phosphotyrosine. We therefore analyzed tyrosine kinases expressed in the K562 chronic myelogenous leukemia cell line, which has a bipotential erythroid and megakaryoblastoid differentiation capacity. Analysis of 357 polymerase chain reaction-amplified cDNA clones led to the identification of 14 different tyrosine kinase-related sequences (JTK1–14). Six of these were identical to previously characterized tyrosine kinases. Clones JTK3, JTK6, and JTK10 probably correspond to the human homologues of similarly identified mouse tyrosine kinase cDNAs, and JTK2 and JTK4 represent novel members of the fibroblast growth factor receptor (FGFR) family. The remaining three clones, JTK5, JTK11, and JTK14 may belong to receptor tyrosine kinases, but lack a close relationship to any known tyrosine kinase. The phylogeny, tissue spesific expression and chromosomal localization of the novel kinases will be discussed.

D 140 Regulation of Jurkat T cell activation by the CD45 protein tyrosine phosphatase.

Jean-Francois Peyron, Sunita Verma and Hergen Spits. DNAX Research Institute. 901 California Avenue. Palo Alto. 94304. CA. USA.

Stimulation of the T cell antigen receptor, TCR-CD3, at the surface of T lymphocytes induces tyrosine phosphorylation of cellular proteins. To explore the possible regulatory role of the CD45 tyrosine phosphatase in this process, we have studied the functional properties of Jurkat T cell variants selected for low or negative CD45 expression, but a normal level of the TCR-CD3 complex. In contrast to normal cells, the CD45 variants secrete strongly reduced levels of interleukin-2 (IL2), IL3 or GM-CSF, after activation by anti-CD3 mAb and the phorbol ester TPA. This defect appears to be at a pretranscriptional level since no IL2 mRNA could be detected and a transiently transfected luciferase gene, directed by the IL2 promoter, could not be activated under these conditions. This defect could be bypassed by stimulation with a calcium ionophore and TPA. The TCR-CD3 complex of the CD45$^-$ variants, upon triggering with anti-CD3 mAb, had only a lower efficiency in stimulation of tyrosine kinase activity and in generation of calcium fluxes which contrasts to the strong inhibition of lymphokines production observed in these cells. This suggests that CD45 may be important for another critical step of the activation process. Activation of the CD45$^-$ variants lead to the transcription of the c-fos protooncogene suggesting that different pathways regulate different activation events.

D 141 EFFECT OF THE TYROSINE KINASE INHIBITOR TYRPHOSTIN ON THE TRANSDUCTION MECHANISMS IN NIH3T3 CELLS,

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We have examined the effect of the tyrosine analog, tyrphostin, which is an inhibitor of the EGF receptor tyrosine kinase on several growth factors mediated events in NIH3T3 cells. Tyrphostin prevents the mitogenic effect mediated by EGF as measured by ^{3}H thymidine uptake. Treatment of NIH3T3 cells for one hour of up to 70 µM tyrphostin does not result in any modifications of neither the level of the c-myc mRNA nor of the S6 kinase activity. A 24 hours drug treatment (40-70 µM) reduced the level of c-myc mRNA although no cytotoxicity was observed at this drug concentration. Experiments are currently in progress to examine the effect of a 24 hour treatment on the EGF dependent activation of S6 kinase.

D 142 ISOLATION AND CHARACTERIZATION OF GENOMIC CLONES CONTAINING THE MURINE C-FGR PROTO-ONCOGENE CODING AND REGULATORY REGIONS, Nitaya Podhipleux and Cheryl L. Willman, Department of Cell Biology, University of New Mexico School of Medicine, Albuquerque, NM 87131
The c-fgr proto-oncogene, a member of the src family of intracellular protein-tyrosine kinases, is selectively expressed in cells of the myeloid lineage. Our previous studies have indicated that c-fgr may play an important role in both the development of the monocytic cell lineage from hematopoietic progenitor cells, and in the functional activation of mature monocytes and macrophages (Willman et al., PNAS 86:4254-4258, 1987; Yi and Willman, Oncogene 4:1081-1087, 1989). Sequences regulating the expression of the murine c-fgr gene have not been previously isolated or characterized. The intron-exon structure of the murine c-fgr gene, paticularly the region encoding the amino terminal domain, is also unknown. To isolate murine c-fgr genomic clones, a lambda GEM-11 mouse liver genomic DNA library was screened with a full-length murine c-fgr cDNA clone (Yi and Willman, Oncogene 4:1081-1087, 1989). Four overlapping phage clones were identified, the largest of which (18kb) contains c-fgr coding exons and 2kb of upstream sequence. An additional 5 kb of upstream sequence is contained in an overlapping phage clone. A complete restriction map of these genomic clones has been generated. Restriction fragments containing the exons encoding the amino terminal domain and the immediate upstream regulatory regions have been subcloned. These regions are currently being sequenced to characterize the murine c-fgr promoter elements and the intron-exon structure of the c-fgr amino terminal domain.

D 143 **Functional analysis of chimeric Insulin/IGF-I receptor molecules.**
Jesper Skou Rasmussen, Asser S. Andersen, Finn C. Wiberg, Thomas Kjeldsen & Niels Peter H. Møller. Molecular Genetics, Bioscience, Corporate Research, Novo Nordisk A/S, DK-2880 Bagsværd, Denmark.
The polypeptide hormone insulin mediates its physiological effects through binding to the insulin receptor. Binding of ligand induces autophosphorylation of the receptor at specific tyrosine residues as a first step in the signal transduction pathway. The IGF-I receptor shows extensive similarity to the insulin receptor in both molecular organisation and signalling mechanism. In particular the tyrosine kinase domain is highly conserved among the two receptors, whereas the transmembrane regions differ.
Recently we have shown that replacing exon 2 & 3 of a soluble insulin receptor with the corresponding IGF-I receptor sequence creates a chimeric receptor with high affinity IGF-I binding and low affinity insulin binding. The ligand binding pattern of the chimeric receptor indicate that the ligand specificity of both receptors reside within exon 2 and 3 of the insulin receptor and the corresponding IGF-I recptor sequence.
A point of central importance is whether such chimeric insulin/IGF-I receptors become activated upon ligand binding and are capable of autophosphorylation. Autophosphorylation of chimeric receptors would indicate a similar mechanism for activation of the receptors.
In the present study three membrane bound chimeric insulin/IGF-I receptors were constructed and expressed in mammalian cells. Ligand binding properties and in vitro capability of autophosphorylation in response to ligand binding will be presented.

D 144 INTERACTIONS OF GAP, GAP ASSOCIATED PROTEINS AND PI 3'-KINASE WITH THE M-CSF RECEPTOR. Michael Reedijk[1,2], Xingquan Liu[1] and Tony Pawson[1]. [1]Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, M5G 1X5, Canada. [2]Department of Medical Genetics, University of Toronto.

The interaction of the macrophage colony stimulating factor (M-CSF) receptor with potential targets was investigated following ligand stimulation of either mouse macrophages, or fibroblasts which ectopically express mouse M-CSF receptors. In both macrophages and fibroblasts, activated M-CSF receptors stimulate PI 3'-kinase, and phosphorylate p62 GAP-associated protein. A mutant M-CSF receptor with a deletion of the non-catalytic kinase insert region was deficient in its association with PI 3'-kinase activity. Phosphorylation of p62 was not markedly affected by this deletion. This mutant retained partial transforming activity in NIH 3T3 cells (Taylor *et al.*, EMBO 8:2029-2037) but was more seriously impaired in Rat-2 cell transformation. These results suggest that the CSF-1R kinase insert, though dispensable for phosphorylation of p62, is required for efficient activation of PI 3'-kinase and full fibroblast transformation. Experiments are in progress to define the regions of the CSF-1R molecule that directly associate with PI 3'-kinase. Preliminary results suggest that recombinant CSF-1R kinase insert molecules form *in vitro* complexes with PI 3' kinase.

D 145 ANALYSIS OF THE MURINE W (*c-kit*), Steel, *c-fms* and *mi* LOCI: THEIR ROLE IN SIGNAL TRANSDUCTION PATHWAY. ROTTAPEL,R. FORRESTER,L., DUBREUIL,P., REITH, A., AND BERNSTEIN, A. Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, M5G 1X5 CANADA.

The *W* locus encodes the proto-oncogene *c-kit*, a member of the receptor tyrosine kinase family that includes the PDGF receptor and the CSF-1 receptor, *c-fms*. The interactions of growth factors with their specific membrane receptors triggger a series of intracellular events that are of critical importance in the regulation of normal cell proliferation. Little is known concerning the gene products and biochem istry of the *c-kit* signal transduction pathway. We describe the use of an *in vitro* assay to analyze *c-kit* function. Normal mast cells can be grown in the presence of IL-3 or on a feeder layer of fibroblasts. The normal expression of a functional *c-kit* signalling pathwayis required for their growth on fibroblasts as mast cells derived from *W* mutants cannot survive in this assay. We have shown that *W* mutant mast cells can survive in this coculture assay if they express exogenous *c-fms* which suggests that the *c-fms* and *c-kit* receptors can stimulate a common downstream mitogenic pathway. We have analyzed mast cells derived from mice carrying the micropthalmic mutation (*mi/mi*) that are also defective in their ability to grow in this coculture system. Unlike the *W* mutant mast cells, exogenous *c-fms* expressed in mi/mi mast cells are unable to complement this defect. This observation suggests that the *mi* gene product may be a downstream subtrate in the *kit/fms* signal transduction pathway.

D 146 AN ANTIBODY AGAINST c-erbB-2 THAT ENHANCES THE CYTOTOXICITY OF CISPLATIN SHARES BIOLOGICAL PROPERTIES OF A LIGAND, Laura K. Shawver, Susan Elliger, Denise J. Raabe, John W. Brandis and Elaina Mann, Department of Molecular and Cell Biology, Triton Biosciences Inc., Alameda, CA 94501.

The c-erbB-2 gene encodes a 185 kDa receptor-like tyrosine kinase with extensive homology to the epidermal growth factor receptor. We have previously shown that an antibody against the c-erbB-2 protein (TAb 250) significantly enhanced the cytotoxic effect of cisplatin on the human breast cell line, SKBR3, and the ovarian cell line, SKOV3. Treatment of SKBR3 cells with TAb 250 alone resulted in a rapid and significant increase in the phosphorylation of gp185. The increase in phosphorylation was alkaline stable, suggesting phosphorylation on tyrosine residues. Rapid receptor endocytosis and degradation have been coupled to elevated tyrosine phosphorylation on other growth factor receptors. Binding analysis using SKBR3 cells showed that treatment with TAb 250 for 40 min resulted in a >10-fold decrease in binding of ^{125}I-TAb 250 compared to untreated cells. This decrease in binding appears to be due to degradation of the receptor as immunoprecipitation of gp185 from whole cell lysate also showed a >10-fold decrease in the amount of protein. The down-modulation of gp185 was dose-dependent and binding of ^{125}I-TAb 250 was restored 24 hr after removal of the antibody. Treatment of SKBR3 cells with the combination of TAb 250 and cisplatin did not alter down-modulation or recovery of the receptor compared to treatment with TAb 250 alone. The effect of TAb 250 on activation of second messengers is currently being examined.

D 147 THE SENSITIVITY OF CELLS TO THE TYROSINE KINASE INHIBITORS ST280 AND ST638 IS INCREASED BY TRANSFORMATION WITH THE V-*SRC* ONCOGENE, Carol G. Shores, Deirdre K. Luttrell, Bartel F. Turk and Tona M. Gilmer, Glaxo Research Labs, 5 Moore Drive, RTP, NC 27709

The viral oncogene v-*src* encodes for a tyrosine specific protein kinase (TK) whose expression rapidly transforms cells both *in vivo* and *in vitro*. ST638 (α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide) and ST280 (3-(3',5'-diisopropyl-4'-hydroxybenzylidene)-2-oxindol) are potent inhibitors of a variety of TKs, including pp60^{v-src}, while having little effect on serine/threonine kinases. We utilized an immortal, nontumorigenic Syrian hamster embryo cell line (10W) transfected with either normal avian c-*src* (4A), or the v-*src* oncogene (61T) to examine ST280 and ST638 effects on cellular phosphotyrosine (ptyr) content. Protein tyrosine phosphorylation was analyzed by Western immunoblotting with a ptyr specific polyclonal antibody. A 6 hour exposure of 61T cells to 20 μM ST280 or 60 μM ST638 led to a dramatic decrease in protein ptyr content, with the tyrosine phosphorylation of 120 and 36 kDal proteins preferentially inhibited by both compounds. These proteins have the same apparent molecular weight as two transformation specific pp60^{v-src} substrates, suggesting that ST280 and ST638 may selectively inhibit the activity of pp60^{v-src} within the cell. In contrast to pp60^{v-src} transformed cells, the ptyr profile of the cells expressing normal pp60^{c-src} was unchanged by a 6 hour exposure to 60 μM ST280 or 100 μM ST638, demonstrating that the compounds preferentially inhibit TK activity in cells expressing pp60^{v-src}. These results indicate that ST280 and ST638 may be useful in elucidating the signal transduction pathway of pp60^{v-src} associated with transformation by the v-*src* oncogene.

D 148 UTILIZATION OF PDGF RECEPTOR MUTANTS TO DETERMINE STRUCTURAL REQUIREMENTS FOR LIGAND BINDING. Laurie M. Strawn, David W. Maher, Daniel J. Donoghue. Department of Chemistry, University of California San Diego, La Jolla, CA 92093-0322.

Interaction of PDGF and v-*sis* protein with their receptor is important in normal and transformed growth. To better understand this interaction, we have expressed a series of mutant forms of the β-type PDGF receptor in Chinese hamster ovary cells. A truncated form of the receptor consisting of only the extracellular ligand binding domain has been expressed and characterized. It is readily secreted and has been partially purified from conditioned media by affinity chromatography on wheat germ agglutinin. To determine the importance of membrane association for ligand binding and receptor dimerization, the ligand binding domain has been expressed on cell surfaces in several different contexts. A truncated form of the β-type receptor which consists of the ligand binding and transmembrane domains has been expressed. Also, the β-type ligand binding domain has been fused to the transmembrane domains from the α-type PDGF receptor and the vesicular stomatitis virus G protein. The role of dimerization in ligand binding will be further investigated with a fusion protein of the ligand binding domain and CSF-1 which dimerizes in the absence of ligand. The affinities for all of these mutant PDGF receptors for PDGF-BB and their abilities to dimerize will be compared to that of the wild type receptor.

D 149 CHARACTERIZATION OF THE TYROSINE PHOSPHORYLATION OF CALPACTIN 1 BY PLATELET DERIVED GROWTH FACTOR.

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Binding of Platelet Derived Growth Factor (PDGF) stimulates the tyrosine kinase activity of the receptor directed toward the receptor itself and other intracellular substrates. Inhibition of tyrosine phosphatases by orthovanadate allows to detect by Western Blot analysis with phosphotyrosine antibodies, several phosphotyrosine-containing proteins after PDGF stimulation. Among these proteins a 39 kDa protein has been recognized as the tyrosine phosphorylated form of the heavy chain of calpactin 1, a protein that binds calcium, phospholipids and actin and that has been shown before to be phosphorylated in tyrosine in cells stimulated by growth factors or transformed by v-src. In vanadate pretreated cells the PDGF-induced phosphorylation of calpactin 1 is slower but much more stable in comparison with other substrates. Moreover the phosphorylation of calpactin 1 occurs at 37°C but not at 4°C. At this temperature receptor internalization does not occur and turnover of phosphoinositides, with the consequent increase in calcium and activation of protein kinase C (PKC), are also inhibited. These data suggest that calpactin 1 is not a direct substrate of PDGF receptor kinase or that other events are required for its phosphorylation, in addition to receptor kinase activation. Membrane association of the phosphorylated form of calpactin 1 depends on the presence of calcium during the extraction. Possible involvement of intracellular calcium and of PKC activation in the regulation of the tyrosine phoshorylation of calpactin 1 are being investigated.

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Supported by A.I.R.C. and by Italian C.N.R. Special Project Signal Transduction.

D 150 CHANGES IN EXPRESSION AND ACTIVITY OF pp60c-src IN DIFFERENT STAGES OF HUMAN COLON TUMORS, Mark S. Talamonti[1], Mark S. Roh[1], and Gary E. Gallick[2], Depts of [1]Surgery and [2]Tumor Biology, M.D. Anderson Cancer Center, Houston TX 77025

In nearly every human colon tumor, the tyrosine kinase activity of $pp60^{c-src}$ is increased greater than 20 fold with respect to adjacent normal colonic mucosa. To determine potential changes which occur during progression, we have examined levels and activity of pp60c-src, and phosphotyrosine-containing proteins at various stages of the disease, focusing on polyps, primary tumors, lymph node and liver metastases obtained from the same patients. In agreement with other laboratories, we observe a 10-20 fold increase in autophosphorylation, and an 8-15 fold increase in phosphorylation of exogenous substrates in colon polyps. Primary tumors were increased 2-4 fold in both kinase activity and protein level with respect to polyps from the same patient. Liver metastases from several untreated patients showed additional increases in expression and activity of $pp60^{c-src}$. These results suggest that an initial change, affecting primarily the activity of the protein occurs during hyperproliferation or at an early stage of tumor development, and additional changes affect the levels of the protein during tumor progression. Additionally, phosphotyrosine proteins from the same specimens were examined by immunoblotting with an anti-phosphotyrosine antibody. A general increase in phosphotyrosine-containing proteins was observed, with specific increases in phosphorylation of a protein of $M_{(r)}$ ~66Kd occurring progressively from polyps to liver metastases.

D 151 ACTIVATION OF THE LCK PROTEIN TYROSINE KINASE BY ANTIGEN PRESENTATION TO MHC RESTRICTED HUMAN T-CELLS
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T-cell activation by the presentation of antigen in the appropriate MHC context involves the phosphorylation of several T cell proteins on tyrosine residues. The responsible T cell protein tyrosine kinase(s) have yet to be identified. We present evidence that enzymatic activation of the abundant T-cell specific protein tyrosine kinase $p56^{lck}$ is an early biochemical event in T-cell activation. We further show that the T-cell phosphorylation events seen following antigen presentation are mimicked by antibody-mediated co-approximation of CD4 with the T-cell receptor/CD3 complex.

D 152 RESPONSIVENESS OF TRITIUM-TRANSFORMED FIBROBLAST TO EPIDERMAL GROWTH FACTOR AND PLATELET-DERIVED GROWTH FACTOR,
Tan-Jun Tong, Ping Huang, Xiao-Lin Liu and Xin-Yuan Shi, Department of Biochemistry, Beijing Medical University, Beijing, China, 100083

The tritium-transformed C3H/10 T1/2 Cl 8 mouse embryo fibroblast(T C3H 10), which could induce tumors in Balb/c nude mice, were not so sensitive to growth stimulation by platelet-derived growth factor(PDGF) and epidermal growth factor(EGF) as their counterpart normal cells (N C3H 10), which was shown by rate of ^{3}H-thymidine incorporation.

The tyrosine-specific phosphorylation of protein in T C3H 10 was manifest while that in N C3H 10 could rarely be measured.

The activity of chromatin-associated protein kinase (CAPK) in C3H 10 was increased more than 50% after transforming. EGF could activate the activity of CAPK in the cultured cells, But the increasing rate in T C3H 10(50.7%) was much lower than in N C3H 10(105.6%).

D 153 EXPRESSION OF ACTIVATED ALLELES OF *abl* IN MURINE BONE MARROW: $P210^{bcr/abl}$ AND $P160^{v\text{-}abl}$ HAVE DIFFERING EFFECTS ON HEMATOPOIESIS,
Richard A. Van Etten, Martin F. Scott, George Q. Daley and David Baltimore, Whitehead Institute for Biomedical Research and Department of Biology, M.I.T., Cambridge, MA 02142 and The Rockefeller University, New York, NY 10021

We have developed a system for the expression of various members of the *abl* oncogene family in bone marrow of mice by a retroviral gene transfer/bone marrow transplantation approach. Expression of the chronic myelogenous leukemia-specific $P210^{bcr/abl}$ protein in this system leads to a variety of hematological malignancies, most prominently a myeloproliferative syndrome with a striking resemblence to the chronic phase of human CML (Daley et al., Science *247*, 824, 1990). Mice with the CML-like syndrome develop a massive overproduction of mature granulocytes, which carry the P210 provirus. The CML-like syndrome is, at least in some cases, a consequence of retroviral infection of the pluripotent hematopoietic stem cell, the disease is transplantable by transfer of bone marrow to syngeneic recipients, and clonally related acute leukemias of both lymphoid and myeloid origin have been observed in secondary transplant recipients, representing evolution of the disease to blast crisis. We have also evaluated the spectrum of disease induced when the viral transforming protein $P160^{v\text{-}abl}$ is expressed in marrow cells by the same vector system in the presence of helper virus. Although recipients of v-*abl*-infected marrow cells demonstrate a moderate granulocytosis early after transplant, lineage analysis has shown that these cells lack the proviral marker, and the animals go on to succumb to provirus-positive pre-B leukemias. Thus, the granulocytosis associated with v-*abl* is reactive rather than a primary malignancy, and different forms of activated *abl* proteins appear to induce distinct disease phenotypes in this system.

D 154 PHOSPHORYLATION OF THE RECEPTOR FOR CSF-1 AT THE C-TERMINUS BY CKII.
Peter van der Geer and Tony Hunter, Molecular Biology and Virology Laboratory, Salk Institute for Biological Studies P.O. Box 85800, San Diego, CA 92186-5800.
The CSF-1 receptor is a member of the protein-tyrosine kinase family and is closely related to the receptors for PDGF and to c-kit and flt. The CSF-1 receptor is the cellular homolog of $gp120^{v\text{-}fms}$, the transforming protein of SM-FeSV feline sarcoma virus. C-fms differs from its transforming counterparts by a series of point mutations and a deletion of the C-terminus. Mutagenesis studies indicate that a point mutation in the extracellular domain (Ser for Leu 301), that renders the protein-kinase activity ligand independent, combined with a point mutation in the C-terminus (Phe for Tyr 973) is sufficient to activate the transforming potential of the CSF-1 receptor. These results strongly implicate deletion of Tyr 973 as an important event in the this activation. In vivo the CSF-1 receptor is phosphorylated to high stoichiometry at the C-terminus on 7-8 different Ser residues, but not on Tyr 973. Fourteen Ser residues are present in the C-terminal most 60 amino acids of the murine CSF-1 receptor. Ten of those are absolutely conserved between human, cat and mouse, and 6 of them are deleted in SM-FeSV v-fms. CKII has been identified as the candidate protein kinase responsible for phosphorylating these sites and we are currently investigating the possible function of these phosphorylation events. Our results suggest that the loss of a series of Ser phosphorylation sites may also contribute to the oncogenic activation of the CSF-1 receptor that results from the deletion of the C-terminus.

D 155 ACTIVATION OF PI-3 KINASE BY VARIANTS OF abl ONCOGENE.
L. Varticovski*, M. Keeler*, G. Q. Daley†, P. Jackson†, D. Baltimore† and L. C. Cantley††.
*Dpt. of Biomedical Research, St. Elizabeth's Hospital, Tufts U., †Whitehead Institute for Biomedical Research, MIT, and ††Dpt. of Physiology, Tufts U. School of Medicine, Boston, MA.
The p210 BCR/abl protein is a result of gene rearrangement and is a hallmark of Ph^+ CML. This chimeric rearrangement results in activation of *abl* oncogene protein-tyrosine kinase activity. The protein-tyrosine kinase activity of *v-abl*, other oncogenes and growth factors is essential for tumorigenicity since tyrosine kinase deficient mutants are not transforming. PI 3-kinase, associates with activated growth factor receptors for PDGF, insulin and CSF-1, and with oncogene products of *src*, *fms*, *yes*, *crk* and polyoma mT. Fibroblast transformation by oncogenes of the *abl* and *src* families requires activation of their protein-tyrosine kinase activity and membrane association via an amino terminal myristoylation. We have demonstrated that PI 3-kinase directly associates with activated protein-tyrosine kinase variants of abl and BCR/*abl* protein. In intact cells, this association leads to accumulation of PI 3-kinase products (PtdIns-3,4-P_2 and PtdIns-P_3) only in myristoylated, transforming variants of BCR/*abl*. These results suggest that myristoylation is required to recruit PI 3-kinase to the site of its substrates at the plasma membrane.

D 156 INHIBITION OF GROWTH FACTOR ACTION BY ANTIESTROGENS IN BREAST CANCER
F. Vignon, H. Rochefort and G. Freiss. INSERM Unit 148 on "Hormones and Cancer", 60 Rue de Navacelles, 34090, Montpellier, France.
Breast cancer cell proliferation is controlled by multiple factors among which steroid hormones (estrogens) and growth factors (IGF-I, EGF) play the major roles. These 2 classes of mitogens acting **via** nuclear steroid receptors and membrane receptors with tyrosine kinase activity are interacting. We have previously shown that antiestrogen 4 OH-Tamoxifen (OH-Tam) can decrease proliferation both by antagonizing estrogen action and by preventing EGF and IGF-I mitogenic activity. We have studied this mechanism by evaluating how OH-Tam affects early steps in growth factor action. In estrogen receptor positive (ER+) cells, OH-Tam time-dependently decreases IGF-I binding by 60%, which could explain the reduction in growth factor mitogenic activity. By contrast, OH-Tam treatment tripled EGF high affinity binding sites, thus suggesting that decreased EGF proliferative action could result of an impaired transducing system. OH-Tam cell pretreatment drastically reduces **in vitro** EGF receptor autophosphorylation reflecting either a decrease in tyrosine kinase activity or the presence of abnormal receptors.
We conclude that antiestrogen OH-Tam may prevent growth factor action in ER+ cells both by modulating the concentration of growth factor binding sites and by altering growth factor receptor functionality.
Supported by the "Institut National de la Santé et de la Recherche Médicale" and the "Association pour la Recherche sur le Cancer"(contract n°6793).

D 157 ACTIVATION OF F_A/GSK3 BY INSULIN IN 3T3-L1 CELLS: COMPARISON BETWEEN PHOSPHATASE-1-ACTIVATING ACTIVITY AND KINASE ACTIVITY.

Emma Villa-Moruzzi, Dip. di Biomedicina Sperim., Infett. e Pubbl., Sez. di Patologia Gen., Univ. di Pisa, Pisa, Italy and John W. Crabb, W. Alton Jones Cell Sci. Center, Lake Placid, NY.

Purified PP1 is activated "in vitro" by two kinase: 1) F_A/GSK3, which phosphorylates PP1 inhibitor-2 (I2) at Thr 72 thus activating PP1, and 2) casein kinase II (CKII), which phosphorylates I2 at Ser 86, 120 and 121; these latter phosphorylations "per se" do not activate PP1 but allow higher activation of PP1 by F_A/GSK3. The aim of the present research if to find whether such mechanism is responsible for PP1 activation by insulin "in vivo". We reported that in 3T3-L1 cells activation of PP-1 by insulin (within 3-5 min) is preceded by reversible activation of F_A/GSK3, assayed as PP1 activator (between 1 and 3 min, peaking at 2 min). Such functional assay is not able to distinguish between the potential contribution of F_A/GSK3 and CKII to activate PP1. For this reason F_A/GSK3 was also assayed using as substrate a 29-aminoacid synthetic peptide (derived from G-subunit of PP1) that includes one site phosphorylated by cAMP-dependent protein kinase (PKA) and two sites that become available to F_A/GSK3 following PKA phosphorylation. The results indicated 1.5-2-fold increase in F_A/GSK3 activity 2 min from exposure to insulin, hence coincident in time with what found by assaying F_A/GSK3 as PP1 activator.

D 158 EGF AND PDGF ACTIVATE PHOSPHOLIPASE C WITHOUT THE INVOLVEMENT OF A G PROTEIN.

Vicentini L.M. and Cattaneo G.,Dept.of Pharmacology,Univ.of Milano and Bologna,Italy

EGF and PDGF,whose receptors possess an intrisic tyrosine kinase (TK) activity,are also capa= ble of stimulating the hydrolysis of phosphoinositides (PI).We have recently shown that in Swiss 3T3 cells PDGF activates phospholipase C (PLC) in a GTP-independent fashion,while bombe= sin behaves like a classical G protein-linked agonist.We have also found that the PLC activa= tion by PDGF is dependent on the TK activity of the PDGF receptor itself,since an inhibitor of the TK activity of the tyrphostin series is capable of antagonizing the PDGF-stimulated (but not the bombesin-stimulated) accumulation of inositolphosphates (IPs).We have now investigated the mechanism whereby activation of the EGF receptor triggers PI hydrolysis in permeable ham= ster fibroblasts where the human EGF receptor has been overexpressed and where receptors for thrombin (a mitogen in these cells coupled to PLC in a pertussis toxin-sensitive manner) are also present.In fact,GTPγS was found to potentiate the coupling of the thrombin receptor to PLC.In contrast,the IPs accumulation was not potentiated but rather inhibited by the simulta= neous presence of GTPγS and EGF as compared to EGF alone.Moreover,the inactive analogue of GTP, GDPβS,inhibited the thrombin- but not the EGF-induced IPs accumulation.Based on our present data on EGF and on our previous data on PDGF,we suggest that the growth factor TK receptors possess a special mode of activating PLC which differs from that used by the seven trans= membrane domain receptors coupled to the enzyme via a G protein.

D 159 FUNCTIONAL ACTIVATION OF THE T CELL ANTIGEN RECEPTOR INDUCES TYROSINE PHOSPHORYLATION OF PHOSPHOLIPASE C, Arthur Weiss and Theresa Kadlecek, Howard Huges Medical Institute, Department of Medicine, University of California, San Francisco, Ca. 94143

The T cell antigen receptor (TCR), a complex 7 chain structure, is known to activate 2 signal transduction pathways: the protein tyrosine kinase (PTK) and the inositol phospholipid (InsP) pathways. The relationship between these two pathways has not been established, although several recent studies have suggested that the PTK pathway regulates the InsP pathway. Studies of growth factor receptors with intrinsic PTK activity have demonstrated their association with phospholipase C (PLC) and the phosphorylation of PLC. Since the TCR activates a PTK, albeit not one intrinsic to the structure of the TCR, we examined the possibility that stimulation of the TCR induces PLC tyrosine phosphorylation. A 10 to 20-fold induction of tyrosine phosphorylated PLC activity could be detected within 15 seconds of stimulation of the TCR on Jurkat cells with anti-TCR monoclonal antibodies. Stimulation of several other cell surface molecules failed to induce tyrosine phosphorylation of PLC. Neither calcium ionophore, phorbol esters, nor their combination induced tyrosine phosphorylation of PLC activity. Recently, we demonstrated that the human muscarinic receptor subtype 1 (HM1), when transfected into Jurkat, could activate PLC independent of the PTK pathway. Stimulation of HM1 failed to induce tyrosine phosphorylation of PLC activity. Thus, phosphorylation of PLC is not absolutely required for all receptor-mediated activation of PLC. Finally, the functional importance of TCR induced PLC phosphorylation was examined using signal transduction mutants derived from Jurkat which fail to activate PLC upon TCR stimulation. J.CaM1 and J.CaM2 have distinct but undefined defects that lead to their inability to activate PLC. J.CaM2 does activate the PTK pathway. J45.01 is a CD45 negative mutant and its TCR is unable to active either the PTK or PLC pathways. When the TCR on these mutants were stimulated, stimulation of tyrosine phosphorylation of PLC was markedly impaired. Collectively, these studies demonstrate that the functional activation of PLC via the TCR, which itself is not a PTK, is associated with tyrosine phosphorylation of PLC.

D 160 A NEGATIVE FEEDBACK LOOP ATTENUATES EGF-INDUCED MORPHOLOGICAL CHANGES. Alan Wells, John B. Welsh, Michael G. Rosenfeld, & Gordon N. Gill. Departments of Pathology and Medicine, University of California, San Diego, School of Medicine, La Jolla, CA 92093-0648.

Activation of the epidermal growth factor receptor (EGFR) by ligand secondarily triggers a host of other cellular enzymes. One of these, protein kinase C (PKC), phosphorylates the EGFR at Thr^{654}. We hypothesize that PKC provides an intracellular negative feedback loop to attenuate EGFR signaling. To test this, scanning electron microscopy was utilized to follow the characteristic EGF-induced retraction of lamellipodia and concommittant cell shape changes. Mutant EGFR were expressed in receptor-deficient NR6 cells. The EGFR were either full length (WT) or carboxyl-terminal truncated at residue 973 (c'973); the latter is resistant to ligand-induced down-regulation. Both the WT- and c'973-expressing cells demonstrated the characteristic lamellipodial retraction after exposure to EGF, with the non-down-regulating c'973 EGFR displaying a more rapid response. Pre-exposure of the cells to TPA blocked this response. Replacement of Thr^{654} by Ala^{654} resulted in EGFR resistant to TPA. Furthermore, cells expressing the Ala^{654} mutations underwent more rapid morphologic changes than the corresponding Thr^{654} EGFR. In the cells expressing Thr^{654} EGFR, down-regulation of PKC by 24 hr exposure to TPA resulted in more rapid EGF-induced changes; the time course was similar to that seen in the Ala^{654} EGFR cells. From these data we deduce that activation of PKC and phosphorylation of the EGFR at Thr^{654} leads to rapid physiological attenuation of EGFR signaling.

D 161 CLONING AND CHARACTERIZATION OF A HUMAN INTESTINE-SPECIFIC cDNA WHICH IS A MEMBER OF THE LIPOCORTIN GENE FAMILY AND MAY BE IMPORTANT IN INTESTINE DEVELOPMENT, Burton M. Wice and Jeffrey I. Gordon, Dept. of Biochemistry and Molecular Biophysics, Washington Univ. School of Medicine, St. Louis, MO 63110

The intestinal epithelium is composed of four principal differentiated cell types: absorptive, goblet, Paneth, and entero-endocrine cells. All of them are derived from a common pluripotent stem cell located in the lower portion of the crypts. Little is known about the molecular mechanisms which regulate the maintenance and growth of these stem cells and how their daughters undergo commitment to and differentiate into the four cell lineages. HT-29 cells are capable of undergoing enterocytic differentiation in vitro. We generated a cDNA library from these cells and by using subtraction hybridization techniques, have isolated a cDNA, Gut-2, whose pattern of expression is consistent with it playing a role in the growth and/or commitment of stem cells. Gut-2 does not appear to code for a differentiation marker since it is expressed at high levels in pre-differentiated HT-29 cells and at lower levels in differentiated cells. Gut-2 is expressed at high levels in the adult human small intestine and was not detected in any of 10 other tissues examined. GUT-2 is also transiently expressed at very high levels in the human fetal intestine during the period of organogenesis and cytodifferentiation. Sequence analysis disclosed that Gut-2 is a new member of the Lipocortin gene family. Lipocortins are substrates for growth factor receptor tyrosine kinases, bind Ca^{++} and phospholipid, and have been implicated in the regulation cellular differentiation. We are currently investigating the role of Gut-2 in regulating the growth and differentiation of intestinal epithelial cells.

D 162 LAR-A TRANSMEMBRANE PROTEIN TYROSINE PHOSPHATASE, Yu, Q. and Weinberg, R.A., Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142

A rat cDNA of LAR-the leukocyte common antigen related gene, which encodes a protein tyrosine phosphatase, has been isolated from a rat fibroblast cDNA library. The 6.8 Kb rat LAR cDNA encodes a protein of 1886 amino acids which has 97% homology to the human LAR protein. Biochemical characterization of the rat LAR protein with antibodies against it indicates that the protein is made as a 190 KD precursor and then is cleaved into at least two smaller products. One of them is a transmembrane protein and its extracellular domain is glycosylated. The sizes and the modifications of the two products are different between normal and transformed cells. The enzymatic activities of the different products in different cells are under investigation. Overexpression of various mutant forms of the LAR cDNA in various cell lines has been carried out to study the biological function of the gene and its protein products in regulation of cell growths

D 163 INHIBITION OF PDGF-INDUCED c-JUN AND c-FOS EXPRESSION BY A TYROSINE PROTEIN KINASE INHIBITOR: CORRELATION WITH DNA SYNTHESIS, Jean Zwiller[1], Paolo Sassone-Corsi[2], Kerry Kakazu[3] and Alton L. Boynton[3], [1]Centre de Neurochimie, CNRS, Strasbourg, France, [2]Laboratoire de Genetique Moleculaire des Eucaroytes, CNRS, Strasbourg, France and [3]Cancer Research Center of Hawaii, Honolulu, Hawaii 96813

The expression of the proto-oncogene c-fos, c-jun, jun-B and jun-D were monitored in quiescent C3H10T1/2 fibroblasts after stimulation with PDGF. The mRNA level of c-fos, c-jun and jun-B, but not of jun-D was stimulated by PDGF. These inductions were abolished when genistein, a specific tyrosine protein kinase (TPK) inhibitor, was added immediately before PDGF, a condition in which DNA synthesis is inhibited. Treatment with PDGF/genistein for 4 hours followed by its replacement with fresh PDGF/genistein-free medium induces the progression of the cells through the G1 phase of the growth-division cycle, without phospholipase C activation (PLC). The removal of PDGF and genistein was accompanied by an important increase in c-fos, c-jun and jun-B mRNA expression, which correlated with the entrance of cells into their G1 phase. Thus, the expression of these proto-oncogenes was obtained in the absence of PLC activation. These results suggest that the mRNA levels of c-jun, jun-B and to the lesser degree c-fos are positively regulated by TPK activity, whereas jun-D is negatively regulated.

Growth Factor Signal Transduction

Growth Signalling: General

D 200 POSTTRANSLATIONAL MODIFICATION AND SUBCELLULAR LOCATION OF THE PROTEIN TYROSINE KINASE $p56^{lck}$, Kurt E. Amrein[+], Andrey S. Shaw[†], John K. Rose[†], Bartholomew Sefton[*], [+] Central Research Unit, Hoffmann La Roche, CH-4002 Basel, [†] Department of Pathology, Yale School of Medicine New Haven, CT 06510, [*] MBVL, The Salk Institute, San Diego, CA 92138

$p56^{lck}$ is a protein tyrosine kinase of the *src* family and is expressed mainly in lymphoid cells. It can interact with the T cell accessory molecules CD4 and CD8 suggesting that it plays an important role in T -cell activation. Two posttranslational modifications are known for $p56^{lck}$, namely myristylation and phosphorylation on serine/threonine and tyrosine. First we studied the effect of myristylation of $p56^{lck}$ on its subcellular location and its interaction with CD4. We found that myristylation of $p56^{lck}$ is important for its association with cytoplasmic membranes; for the interaction with CD4 myristylation is helpful but not crucial. Altering the subcellular location of $p56^{lck}$ has no effect on the tyrosine phosphorylation of $p56^{lck}$; the serine phosphorylation however is changed in mutant forms of $p56^{lck}$ expressed either in the cytoplasm or directed to the nucleus with the help of a nuclear targetting signal hooked up to the aminoterminus of the kinase.

D 201 EPIDERMAL GROWTH FACTOR RECEPTOR PHOSPHORYLATES PROTEIN 4.1 AND MODULATES ITS FUNCTION IN VITRO, Richard A. Anderson, G. Subrahmanyam, and Paul J. Bertics, Departments of Pharmacology and Physiological Chemistry, University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706

Protein 4.1 was initially characterized as a protein that regulates cytoskeletal assembly in erythrocytes. However, recent studies have shown that protein 4.1 is ubiquitous to all cells and that the diversity in protein 4.1 species is due to alternative m-RNA splicing. Here, we show that purified erythrocyte protein 4.1 was phosphorylated in vitro by EGF-receptor tyrosine kinase. Phosphoamino acid analysis of phosphorylated protein 4.1 showed only phosphotyrosine, and partial proteolysis of phosphorylates protein 4.1 showed that the 30 kDa and 8 kDa regions contained the phosphorylated sites. A time course of phosphorylation coupled with limited proteolysis suggested that the 8 kDa region was the preferred site for phosphorylation. The 8 kDa region is required for assembly of the spectrin-actin-protein 4.1 ternary complex. When stoichiometric amounts of phosphate were incorporated, this reduced protein 4.1's ability to promote assembly of the spectrin-actin-protein 4.1 ternary complex. One of the early events in EGF-stimulated mitogenesis is reorganization of the cytoskeleton. The results presented here suggest that tyrosyl phosphoryla-tion of protein 4.1 may regulate cytoskeletal networks in cells. In contrast to erythrocytes, A-431 cells have at least four different forms of protein 4.1. Currently, we are investigating the in vivo phosphorylation of protein 4.1 in A-431 cells.

D 202 REGULATION OF THE HUMAN β-GLOBIN GENE, M. Antoniou, F. Grosveld, E. deBoer, N. Dillon, P. Fraser, D. Greaves, O. Hanscombe, J. Hurst, M. Lindenbaum, S. Philipsen, S. Pruzina, D. Talbot and D. Whyatt, Laboratory of Gene Structure & Expression, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

The human β-globin gene family is located on the short arm of chromosome 11 and contains five functional genes. These are arranged in the same order as they are expressed during development, i.e. $5'-\epsilon-\gamma_G-\gamma_A-\delta-\beta-3'$ over a distance of 55kb. The embryonic ϵ-globin gene is active when the yolk sac is the hematopoietic tissue, the γ-globin genes are active in the liver during the foetal stage, and the δ- and β-globin genes in the adult stage bone marrow (for review, see Collins & Weissman, 1984). Each gene contains a number of tissue- and developmental stage-specific regulatory regions and the entire locus is controlled by the so-called Dominant Control Region (DCR). This DCR consists of four *strong hypersensitive regions (HSS) upstream of the ϵ-globin gene. Addition of these* regions confers copy number dependent expression on the human β-globin gene in murine erythroleukaemia cells and transgenic mice, at levels comparable to the endogenous mouse globin genes. We describe a deletional analysis of three of these hypersensitive regions and show that 200-300bp fragments are sufficient to direct copy number dependent, integration site independent expression of the human β-globin gene. Biochemical analysis in vitro and mutagenesis experiments in vivo show at least two erythroid specific proteins (NF-E1 and NF-E2) and one non- erythroid protein to be essential for the function of this region. Addition of the DCR to globin genes also results in altered developmental expression patterns of an individual gene. By using multiple globin genes, we show that the combination and order of genes is important for their expression. A model for the regulation of this multigene locus will be presented.

D 203 ASSEMBLY AND REGULATION OF TWO DISTINCT PHOSPHATIDYLINOSITOL-4-PHOSPHATE 5-KINASES ON NATIVE MEMBRANES, Chantal E. Bazenet, David L. Harris, and Richard A. Anderson, Department of Pharmacology and the Cell and Molecular Biology Program, University of Wisconsin Medical School, 1300 University Avenue, Madison, WI 53706
The human erythrocyte membranes contain two structurally distinct PIP kinases: the type I (membrane-bound) and the type II (both cytosolic and membrane-bound) (1). Although both PIP kinases are active toward PIP micelles and PIP in liposomes, only the type I is active toward intrinsic PIP in erythrocyte membranes (1). The activity of the type I PIP kinase on inside-out membrane vesicles (IOVs) is stimulated by increased membrane phosphatidylinositol-4-phosphate (PIP) and inhibited by increased phosphatidylinositol-4,5-bisphosphate (PIP_2). Regulation of the type I PIP kinase by the ratio PIP/PIP_2 is a property intrinsic to the native membranes and does not occur on liposomes. In native membranes, intrinsic PIP is not accessible to the type II PIP kinase; however, increased PIP above 1 mole percent was phosphorylated by the type II PIP kinase. Intrinsic PIP and exogenous PIP below 1 mole percent may be sequestered as well as PIP_2 since PIP_2 below 1 mole percent does not inhibit PIP kinase activity. All these data suggest that PIP kinases on native membranes are regulated by sequestration of PIP and PIP_2 and by membrane components which sense the ratio of PIP/PIP_2. Such a mechanism may explain plasma membrane localization and the stimulation of PIP kinase activity upon agonist activation of second messenger production.
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D 204 **EXPRESSION OF THE INT-1 RELATED PROTEIN (IRP/WNT-2) INDUCES TRANSFORMATION IN MAMMARY EPITHELIAL CELLS.** Andrew Blasband and Jackie Papkoff, Syntex Research, 3401 Hillview Ave, Palo Alto, Calif., 94304
The *int-1/Wnt-1* gene family consists of several members, including *int-1/Wnt-1* and *irp/Wnt-2*. The human *irp/Wnt-2* gene (h-*irp/Wnt-2*) was identified in a search for the cystic fibrosis gene, it is linked to the cystic fibrosis locus and expressed in lung. The overall amino acid identity between h-irp/Wnt-2 and m-int-1/Wnt-1 is 38% and includes conservation of structural features and 22/23 cysteine residues. *irp/Wnt-2* mRNA is normally expressed in adult lung and heart and in fetal development in allantoic mesoderm, lung and heart. An irp/Wnt-2 protein has not been identified and it is not known if irp/Wnt-2, like m-int-1/Wnt-1, can participate in neoplasia. To address these questions, inducible expression vectors for m-*int-1/Wnt-1* or h-*irp/Wnt-2* cDNAs were constructed and transfected into either CHO cells for biochemical studies or into the mouse mammary epithelial cell line, C57mg, to assess biological activity.

Immunoprecipitations of ^{35}S-cysteine labeled cell extracts from h-*irp/Wnt-2* transfected CHO cell lines, using an anti-peptide antibody, yielded two proteins of approximately 33,000 (33K) and 35,000 (35K) daltons. h-irp/Wnt-2 protein has at least one N-linked glycosylation since tunicamycin treatment of the cells blocked the appearance of the 35Kd species whereas the 33Kd protein was unaffected. Comparative pulse-chase studies between m-*int-1/Wnt-1* and h-*irp/Wnt-2* transfected CHO cell lines demonstrated that, like m-int-1/Wnt-1, h-irp/Wnt-2 moves inefficiently through the secretory pathway. In other studies we have shown that secreted m-int-1/Wnt-1 protein is released into the culture medium only after suramin treatment of the cells. Similarly, a small amount of h-irp/Wnt-2 protein was detected in the culture fluid of transfected CHO cells after suramin treatment. These data indicate that h-irp/Wnt-2, like m-int-1/Wnt-1 adheres to the cell surface or extracellular matrix.

C57mg cells have previously been used to assess the transforming properties of the m-*int-1/Wnt-1* gene. We have found that transfection of this cell line with h-*irp/Wnt-2* results in loss of contact-inhibited growth and a transformed phenotype in monolayer culture, similar to the effects of m-*int-1/Wnt-1*. These studies with h-*irp/Wnt-2* suggest that it shares many biological and biochemical features in common with m-*int-1/Wnt-1*.

D 205 ACTIVATION OF CASEIN KINASE II BY PHOSPHORYLATION IN RESPONSE TO EPIDERMAL GROWTH FACTOR, Cochet C., Balogh A., Chambaz E.M., Filhol O.,Unité INSERM 244, DBMS, BRCE, CEN.G, 85X, 38041 Grenoble Cedex, France.
Signal transduction in response to growth factors seems to involve a cascade of protein kinases. Among them, casein kinase II is of particular interest because phosphorylation studies indicate that this enzyme phosphorylates a large spectrum of nuclear proteins including enzymes that modify DNA topology and several transcription factors. Recent evidence indicates that casein kinase II activity is modulated by the action of several polypeptide hormones including insulin (1), insulin like growth factor I (2) and EGF (3). Localization studies show that in serum-deprived cells, the enzyme is found in the cytoplasm. Upon serum or FGF addition, the enzyme concentrates in the nucleus (4). Although the mechanism of this nuclear translocation is not understood, data indicate that the transient stimulation of casein kinase II (CKII) in response to growth factors depends on phosphorylation events.
We describe the properties and partial purification of a serine/threonine protein kinase whose activity is enhanced 5-fold within 5 min of exposure of A431 cells to EGF. This protein kinase can be recovered in anti-phosphotyrosine antibody immunocomplexes after EGF treatment of A431 cells. We found that the isolated kinase phosphorylates and stimulates by 3-fold CKII activity. This short-term regulation of CKII by growth factors points a possible role of CKII as an intermediate step in the transduction of signals from growth factor receptors to effector proteins within the cell nucleus.
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4. Filhol O., Cochet C., Chambaz E.M., Biochemistry (in press).

D 206 SUBCELLULAR LOCALIZATION OF THE RAS-RELATED RAP1 AND RAP2 PROTEINS,
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The ras-related rap1 and rap2 proteins are 21kD GTP-binding proteins that exhibit approximately 50% identity with ras proteins and share a similar organization of their structural domains. Because ras and rap proteins have the same sequence in their effector regions, it has been proposed that they might have antagonistic functions within the cell; indeed, overexpression of the rap1A/Krev-1 gene has been shown to revert the phenotype of K-ras transformed cells. Similarly to ras proteins and nuclear lamins, rap proteins posess a C-terminal CAAX sequence that specifies their modification by a polyisoprenoid derivative and association with cellular membranes; in addition, the rap2 protein is further modified by a palmitate moiety on cysteine residues upstream from the CAAX box. Using affinity-purified polyclonal rabbit antibodies we show by indirect immunofluorescence that the rap1 protein localizes to a perinuclear area that is stained by antibodies specific of the Golgi complex. Furthermore, upon subcellular fractionnation of human epidermoid carcinoma HEp2 cells, the rap1 protein is only found in the Golgi-enriched fractions. In contrast, the rap2 protein is located in a more diffuse and reticulated structure that was identified as the endoplasmic reticulum. Neither protein colocalizes with ras proteins at the inner face of the plasma membrane. Possible mechanisms involved in the differential localization of these closely related proteins will be discussed in relation with their possible physiological functions.

D 207 **DIFFERENTIAL BINDING OF GUANINE NUCLEOTIDE ANALOGUES TO LOW MOLECULAR MASS GTP-BINDING PROTEINS,**
Burton F. Dickey, Timothy M. Shannon, Jordan B. Fishman, Jeffrey B. Rubins, George E. Wright and Timothy P. Noonan, Departments of Cell Biology and Pulmonary Medicine, Baylor College of Medicine, Houston TX, and Department of Pharmacology, University of Massachusetts Medical Center, Worcester MA

GTP-binding proteins have been likened to "molecular switches" which regulate the activity state of a wide variety of biochemical processes. It is possible to lock these switches in the active triphosphoguanosine-liganded conformation with slowly hydrolyzed GTP analogues such as GTPγS, but without protein specificity. The binding of [α32P]GTP to four GTP-binding proteins of molecular masses 26, 25, 24, and 23 kDa, partially purified from bovine lung lamellar bodies and immobilized on nitrocellulose, was competed with a series of GTP analogues with modifications at multiple sites. One analogue, N^2-(p-n-butylphenyl)guanosine 5'-(β,γ-difluoromethylene)triphosphate, showed an approximately 50-fold higher affinity for the 25 kDa protein compared to the others. Analysis of each modification in the synthetic guanine nucleotide revealed that virtually all of the specificity was conferred by the β,γ-difluoromethylene group, and further, that most of that specificity resided in the β,γ-methylene rather than in the fluorines. These results show that despite the high degree of homology among GTP-binding proteins in the GTP binding and hydrolyzing regions, subtle differences can be exploited to confer differential binding on synthetic nucleotides.

D 208 PHOSPHOLIPASE C-CATALIZED HYDROLYSIS OF PHOSPHATIDYLCHOLINE IS A CRITICAL STEP FOR MATURATION OF XENOPUS LAEVIS OOCYTES IN RESPONSE TO INSULIN ras p21,
Isabel Dominguez[1], Antonio García de Herreros[1], Maria T. Diaz-Meco[1], Grazia Graziani[2], Per Henrik Guddal[3], Terje Johansen3, Jorge Moscat[1*] [1]Medicina y Cirugía Experimental, Hospital General "Gregorio Marañón", Dr.Esquerdo 46, 28007 Madrid, Spain; [2]Laboratory of Cellular and Molecular Biology, National Cancer Institute, NIH, Bethesda, Maryland 20892, USA; [3]Institute of Medical Biology, University of Tromso, 9001 Tromso, Norway.

Recent studies have demonstrated the activation of phospholipase C-mediated hydrolysis of phosphatidylcholine both by growth factors and by the product of ras oncogene, ras p21. Also, evidence has been presented indicating that the stimulation of this phospholipid degradative pathway is sufficient to activate mitogenesis in fibroblasts. In Xenopus laevis oocytes, microinjection of transforming ras p21 is a potent inducer of maturation, whereas microinjection of a neutralizing anti-ras p21 antibody specifically inhibits maturation induced by insulin but not by progesterone. The results presented here demonstrate that phosphodiesterase-mediated hydrolysis of phosphatidylcholine is critically involved in the maduration pathway activated by insulin/ras p21 but not by progesterone.

D 209 EXPRESSION OF A NOVEL PDGF RECEPTOR ASSOCIATED PROTEIN, P85 THAT REGULATES THE PI3 KINASE ACTIVITY ASSOCIATED WITH THE PDGF β-RECEPTOR.
J.A. Escobedo, W.M. Kavanaugh and L.T. Williams. Howard Hughes Medical Institute, Cardiovascular Research Institute, University of California, San Francisco, CA., 94143.
Tyrosine-phosphorylated PDGF receptors bind and phosphorylate several proteins of the signal transduction pathway. This modification of some of these molecules such as PLC-γ, GAP and raf-1 appears to regulate their activities. Using an in vitro association system we have identified two additional receptor-associated proteins of 85kD and 110kD present in immunoprecipitates of activated receptors. These proteins associate with the receptor at a defined amino acid sequence in the kinase insert region that contains site(s) of tyrosine phosphorylation. Deletion of the kinase insert region prevents these associations. Similarly a 20 amino acid peptide phosphorylated at position Y708 or Y719 blocks the asssociation of 85kD/110kD protein with the receptor. Previous evidence has suggested that the 85kD protein is the molecule encoding a PI3 kinase activity. Using immobilized phosphorylated receptors as an affinity reagent we purified the 85 kD protein from 3T3 cell lysates. The 85kD protein contains two SH2 domains. The recombinant 85kD protein expressed in mammalian and insect cells binds to the receptor with the same characteristics as the 85kD protein from 3T3 cells. Overexpression of this protein in mammalian cells inhibits the endogenous PI3 kinase activity associated with activated PDGF receptors. This suggests that the 85kD protein is not the catalytic subunit of the PI3 kinase but is either a regulatory subunit of PI3 kinase or a competitive antagonist that binds to the same receptor sequence as PI3 kinase.

D 210 Bombesin Receptor Heterogeneity, Solubilization, and Antagonists.
Bradford O. Fanger, James J. Knittel, Elizabeth A. Cashman, John L. Krstenansky, and J. Vincent Edwards. Merrell Dow Research Institute, Cincinnati, OH 45215-6300.
Bombesin, gastrin releasing peptide (GRP), and litorin are homologous GRP receptor agonists. These peptides bind a single class of sites on murine pancreatic membranes with a K_d = 150 pM, 20 pM, and 150 pM, respectively. The murine pancreatic receptor is a 70 kDa membrane protein linked to a G-protein which stimulates phosphatidylinositol phosphate (PI) turnover. ^{125}I-GRP-receptor complexes were formed in the absence and presence of various competing peptides and then solubilized with 0.5% digitonin. Binding was assayed with G-50 spin columns. The binding specificity of the membrane receptors was maintained after solubilization since only the analogs with an intact C-terminal receptor binding domain competed with ^{125}I-GRP. ^{125}I-GRP was dissociated by nonhydrolyzable GTP analogs indicating that G-proteins were associated with the solubilized receptor complexes.

Several novel bombesin receptor antagonists were synthesized. The N^{α}-octyl-Gln-Trp-Ala-Val-D-Ala-His-Leu-NH_2 analog had the highest affinity (K_d = 6 nM) of the N^{α}-alkyl series acetyl (K_d = 100 nM), lauryl (K_d = 400 nM), and palmityl (K_d = 500 nM). The octyl analog was the most potent antagonist of GRP-stimulated PI turnover; none of these analogs exhibited agonist activity. The pseudopeptides [$Phe^8\psi[CH_2S]Leu^9$]litorin and [$Phe^8\psi[CH_2S(O)]$-Leu^9]litorin bound to the receptor with a K_d of 1-5 nM. These analogs were also pure antagonists with a higher potency than the related $\psi[CH_2NH]$ compound. Interestingly, [$Phe^8\psi[CH_2N(CH_3)]Leu^9$]litorin was an agonist. Its binding was indicative of two classes of receptor sites, suggesting receptor heterogeneity.

D 211 MULTI-SITE PHOSPHORYLATION OF P190MET TYROSINE KINASE
Riccardo Ferracini, Paola Longati and Paolo M. Comoglio. Department of Biomedical Sciences and Oncology, University of Torino, Italy.
The MET protooncogene encodes for a tyrosine kinase protein of 190kd (p190MET). It is a transmembrane glycoprotein structurally related to the growth factor receptor family featuring two disulfide linked subunits of 150 (β) and 50 (α) kd. Previous studies showed that in the gastric carcinoma cell line GTL16 the MET gene is amplified and overexpressed and the p190MET protein is constitutively phosphorylated on tyrosine. To understand the role phosphorylation plays in regulating the kinase activity of p190MET we have studied the major tyrosine phosphorylation sites of this kinase. We have compared the phosphorylation status of the MET protein from overexpressing cells and from cells expressing normal amounts of p190MET. GTL16 and CALU 1 cells were labelled in vivo with $^{32}P_i$-orthophosphate and immunoprecipitated with an anti-p190MET antiserum raised against a synthetic peptide from the C' terminus of the protein. The $^{32}P_i$-labelled p190MET protein was subjected to CNBr fragmentation and trypsin digestion. The major resulting phosphopeptides were analyzed on borate gel electrophoresis, thin layer 2D electrophoresis and RPC HPLC. They corresponded to the ones identified after in vitro phosphorylation of the p190MET protein. The kinetics of phosphorylation was studied in vitro for the various phosphorylation sites. A different time course of phosphate incorporation was observed for different phosphopeptides. Prediction studies of the tyrosine phosphorylation sites was performed using Edman degradation analysis and synthetic peptides designed as tyrosine containing substrates for autophosphorylation.

D 212 THE MITOGEN/ONCOGENE-ACTIVATED S6 KINASE: IDENTIFICATION OF THE PHOSPHORYLATION SITES, Stefano Ferrari, Sara C. Kozma and George Thomas, Friedrich Miescher-Institut, PO Box 2543, 4002 Basel, Switzerland

The addition of growth factors to quiescent Swiss 3T3 cells results in the ordered phosphorylation of five serines which are clustered at the C-terminus of the 40S ribosomal protein S6. This process is thought to modulate the activation of protein synthesis, an early obligatory step in the mitogenic response. The kinase responsible for regulating this step in mitogen/oncogene-activated cells and in liver of rats injected with cycloheximide has been identified and purified to homogeneity. The S6 kinase is a component of a phosphorylation cascade and its activity is regulated *in vivo* by phosphorylation on serine and threonine. Tryptic digestion of the S6 kinase yielded a number of peptides which were resolved by means of narrow-bore reverse phase chromatography on an inert HPLC system. Partial aminoacid sequences were obtained from 17 of the resulting peptides, accounting for a total of 200 residues. Based on the aminoacid sequence, a specific DNA fragment was obtained by means of PCR. This fragment was used to screen a rat liver cDNA library. One of the positive clones was sequenced and shown to encode a protein homologous to serine/threonine kinases. We identified one of the phosphorylation sites while sequencing tryptic peptides. Due to the low abundance of the molecule the strategy for the identification of the remaining sites is to: (i) mix *in vivo* 32P-labeled S6 kinase purified from hepatocytes with homogeneous S6 kinase from rat liver; (ii) generate tryptic maps, and (iii) sequence 32P-labeled peptides. Mutation of single sites of phosphorylation will allow us to establish their role in the process of S6 kinase activation.

D 213 NUCLEAR TRANSLOCATION OF CASEIN KINASE II IN RESPONSE TO SERUM OR GROWTH FACTORS : CHARACTERIZATION OF ITS NUCLEAR BINDING SITES, Filhol O., Cochet C. and Chambaz E.M, Unité INSERM 244, DBMS, BRCE, CEN.G, 85X, 38041 Grenoble Cedex, France.

Casein kinase II (CKII) is an ubiquitous protein kinase whose precise cellular functions and mechanisms of control remain to be clarified. Using both immunocytochemical localization and biochemical assay of the enzymatic activity, it was observed that CKII concentrates in the nuclear compartment of adrenocortical cells activated to proliferate. In vitro studies disclosed that nuclear preparations accumulate and tightly bind purified CKII in an ATP and temperature-dependent process. These findings strongly suggest that a cytoplasmic-nuclear CKII shuttle may take place in intact cells and may participate in the regulation of nuclear activity in response to cell growth or differentiation signals (1). In order to further characterize the nuclear components interacting with the kinase, a combination of saline extraction SDS and DNase treatments were carried out, suggesting that both CKII-protein and CKII-DNA interactions were involved. CKII associates with high affinity to nuclear proteins, some of them most likely representing substrates of the kinase. In addition, CKII binds to DNA in vitro (resulting in the stabilization of the kinase activity) and is found associated with DNA following its accumulation into nuclei. These results strongly suggest that nuclear accumulated CKII may be a component of specific DNA protein complexes, possibly taking part in the regulation of the transcription machinery, and of special potential interest in the response to mitogenic extracellular signals.

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J. Biol. Chem., 1990, 265, 7709-7712.

D 214 ROLE OF PHOSPHOTYROSINE PHOSPHATASES IN THE ACTIVATION OF HUMAN T CELLS: EVIDENCE THAT CONTINUES ACTIVITY OF A TYROSINE PHOSPHATASE IS REQUIRED FOR TcR ASSOCIATED TYROSINE KINASE ACTIVITY Mary C. Fletcher*, Lawrence E. Samelson#, Carl H. June*
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In previous studies we have shown that the earliest measurable event following the interaction between the CD3?TcR and monoclonal antibodies directed against this complex is the increased incorporation of phosphate into tyrosine-containing proteins. More recent studies other laboratories indicate a requirement for the CD45 phosphotyrosine phosphatase CD45 in inititation of TcR, but not IL-2, mediated signal transduction.

The following observations were made when T cells were treated with Phenylarsine Oxide (PAO), a phosphotyrosine phosphatase inhibitor. (1) Treatment with PAO, or another PTP Na_3VO_4, caused small increases in phosphotyrosine incorporation into T cell substrates, whereas PAO and Na_3VO_4 together produced a synergistic increase in phosphotyrosine.
(2) PAO pretreatment followed by anti-CD3 MAb yielded a synergistic increase in the incorporation of phosphate into tyrosine residues at concentrations of PAO below 10 uM.
(3) Treatment with anti-CD3 MAb followed by PAO 90 seconds later, at the time of peak production of Inositol-1,4,5-trisphosphate (IP_3), ablated IP_3 production. We conclude that continuous PTP activity is required for initiation and maintenance of TcR mediated signal transduction.

D 215 PTYR-PROTEINS CHARACTERISTIC OF CHRONIC MYELOGENOUS LEUKEMIA

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The presence of an aberrantly active Abelson protein-tyrosine kinase ($p210^{bcr\text{-}abl}$) is characteristic of chronic myelogenous leukemia(CML) and clearly involved in the development of this disease. An attractive scenario has the $p210^{bcr\text{-}abl}$ tyrosine phosphorylating a key cellular substrate of a growth-factor receptor, thereby tricking hematopoetic stem cells into proliferative responses.

We have previously reported that CML cells contain several phosphotyrosyl proteins that are particularly interesting in that they appear to be found in all CML cell lines and patients' cells that we have examined. One of these proteins appears to be the major protein that becomes tyrosine phosphorylated in human cells exposed to colony stimulating factor-1 (CSF-1) and to EGF. These proteins share the same molecular weight (56-kDa) on SDS polyacrylamide gel electrophoresis and are indistinguishable by isoelectric focusing and by phosphopeptide maps. As an initial part of an effort to further characterize these proteins and their role in cell growth, we have developed monoclonal antibodies specific for the CML 56-kDa protein(s).

Another CML protein appears identical to a 62-kDa protein that becomes tyrosine phosphorylated in A431 cells exposed to the growth factor, EGF. This 62-kDa protein forms macromolecular complexes with proteins containing amino acid sequences homologous to the SH2 domain of the src tyrosine kinase. One of these associating proteins is GAP (GTPase activing protein), which regulates the GTPase activity of the ras oncogene product.

D 216 ASSEMBLY AND SIGNALLING THROUGH FcγRIIIA IN NK CELLS.

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The receptor for IgG immune complexes on NK cells (FcγRIIIA) demonstrates antibody-dependent cellular cytotoxicity (ADCC) upon crosslinking with immune complexes mediated by increases in PI-turnover and Ca^{2+} influx. The receptor is composed of at least 3 subunits: The α subunit interacting with immune complexes and the γ and ζ chains first described as components of the high affinity IgE receptor and the T cell receptor (TCR) respectively. These subunits are involved in receptor assembly and signal transduction. Despite the requirement for ζ chain in the assembly of both FcγRIIIA and TCR the mechanism by which the subunit mediates surface expression was found to differ. In addition to its role in assembly ζ is a component of the signalling pathway by FcγRIIIA in NK cells. The role of protein phosphorylation and the kinases involved will be discussed.

D 217 MITOGEN DEPENDENT CHANGES IN PROTEIN PHOSPHOTYROSINE PHOSPHATASE ACTIVITY IN HUMAN T-LYMPHOCYTES.

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Tyrosine phosphorylation is thought to be an important regulatory mechanism in the control of cell growth and proliferation. The level of phosphotyrosine in the cell is the net result of the activities of both specific tyrosine kinases and phosphatases. Phosphotyrosine phosphatase activity, in subcellular fractions from unstimulated and 3 day PHA-stimulated human T-lymphocytes, was assayed and compared to tyrosine kinase activity in the same fraction. Phosphotyrosine phosphatase activity was found in all fractions isolated. PHA stimulation resulted in an increase in total phosphatase activity in the cells roughly proportionate to the increase observed in tyrosine kinase activity. Tyrosine phosphatase activity in the plasma membrane fraction increased in parallel with the kinase activity. In contrast to the characteristic activation dependent increase in kinase activity associated with the detergent insoluble cytoskeletal fraction, phosphatase activity decreased in this fraction. The cytoskeleton was the only fraction in which a coordinated change in kinase and phosphatase activity was observed which might result in a direct increase in phosphotyrosine levels.

D 218 FUNCTIONAL REGIONS OF A PROTEIN KINASE IDENTIFIED BY RATIONAL SCANNING MUTAGENESIS, Craig S. Gibbs and Mark J. Zoller, Department of Protein Engineering, Genentech Inc, 460 Point San Bruno Blvd, South San Francisco, CA 94080.

A systematic mutagenesis strategy, charged-to-alanine scanning mutagenesis combined with in vivo and in vitro screening for functional defects, was applied to identify the functional regions and residues in the catalytic subunit (C_1) of Saccharomyces cerevisiae cAMP-dependent protein kinase. The analysis of mutants by steady-state kinetics and the construction of additional site-directed mutants allowed the identification of residues important in catalysis, in determining specificity for peptide and MgATP substrates and in the recognition of the regulatory subunit. The data suggests that the catalytic domain of the can be divided into two functional domains, a small N-terminal domain that interacts with MgATP and a large C-terminal domain that interacts with the recognition sequence flanking the phosphorylation site in protein and peptide substrates. Catalytic residues were identified in both domains suggesting that the active site might be formed at the interface between these two domains. The properties of mutants defective in substrate specificity were consistent with an ordered reaction mechanism.

D 219 A DOMINANT NEGATIVE ras MUTANT BLOCKS POSSIBLE TARGETS FOR SIGNAL TRANSDUCTION BY TYROSINE KINASE ONCOGENES. Lourdes Gutierrez, Yoshifumi Ogiso, Linda S. Wrathall, Yu-Wen Hwang and Thomas Y. Shih, Laboratory of Molecular Oncology, National Cancer Institute, Frederick, MD. 21702-1201, and New York State Institute for Basic Research in Developmental Disabilities, Staten Island, New York, 10314 (Y.-W. H.)

Oncogenes are activated version of cellular protooncogenes that control cell growth and differentiation. It is believed that these genes participate in signaling pathways. We have identified several dominant negative ras mutants capable of inhibiting c-ras proto-oncogene function (N116Y or I mutations of v-H-ras). The 116Y mutant blocks transformation by retroviruses carrying protein-tyrosine kinase oncogenes including v-abl, v-fes/flp, v-src, v-fms and v-sis. Transformation by the homologous v-H-ras, v-K-ras and v-bas, is not affected suggesting that ras mutant blocks an upstream target of c-ras protooncogenes required for PTK oncogene transformation. We have established a series of NIH3T3 cell lines to investigate the biochemical targets and signaling pathways mediated by ras . Preliminary experiments showed that protein kinase C pathway was disrupted in the presence of 116Y ras mutant. Recent studies have shown that ras GAP is present in a protein complex after stimulation with PDGF. Here we found a decrease in the total amount of GAP in 116Y cells. Sequestration of ras targets could be an explanation for the reduction. Studies are in progress to determine the biochemical targets of inhibition by the dominant negative ras mutants.

D 220 **Three H1 Kinase Activities From a Mammalian CDC2 Mutant**, Joyce R. Hamaguchi, Jon Pines*, Tony Hunter*, and E. M. Bradbury, Biological Chemistry, University of California School of Medicine, Davis, CA 95616 and *The Salk Institute, Dept. of Molecular Biology/Virology, La Jolla, CA 92037.
The mouse FT210 cell line is a G2 phase temperature-sensitive mutant with a defect in the CDC2 gene (J. Th'ng *et al* (1990) Cell, in press). At the nonpermissive temperature of 39°, synchronized FT210 cells progress through G1 and S phases and block in G2 phase. Histone H1 undergoes normal G1/S phosphorylations but G2/M associated hyperphosphorylation of H1 does not occur. Thus, G1/S phase H1 kinase remains active at the nonpermissive temperature and must be distinct from the H1 kinases that contain defective $p34^{cdc2}$. We have identified three distinct histone H1 kinase activities from asynchronous cultures of FT210 cells and the parent cell line, FM3A. *In vitro*, two of the kinases isolated from FT210 cells are thermolabile at 39° whereas the third kinase is stable. Immunoprecipitation protocols were employed to verify the distribution of $p34^{cdc2}$ among the temperature-sensitive kinases and to investigate the presence of the cyclin proteins A and B. The results indicate that $p34^{cdc2}$ forms two thermolabile complexes in FT210 cells, one with cyclin A and one with cyclin B. The thermostable H1 kinase contains cyclin A and a kinase catalytic subunit that is not recognized by antiserum directed against the C-terminal sequence of $p34^{cdc2}$. We propose that the G1 and S phase H1 phosphorylation events are mediated by this thermostable kinase and that it is homologous with the cyclin A-p33 kinase recently identified in HeLa cells (J. Pines and T. Hunter (1990) Nature, 346, 760). In addition, the G2/M associated hyperphosphorylation of H1 requires a kinase containing $p34^{cdc2}$ complexed with either cyclin A or cyclin B.

D 221 SIGNAL TRANSDUCTION IN MURINE T HELPER CELLS IN RESPONSE TO IL-1,
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Murine CD4$^+_-$ T helper cells can be divided into two types, according to their pattern of lymphokine production; namely, Th1 cells secrete IL-2 and γ-INF, while Th2 cells produce IL-4 and IL-5. Another distinction between these two Th cell subsets is that only Th2 cells express high levels of p80 IL-1R and are dependent on IL-1 for induction of proliferation in response to antigen. We have analyzed the signal transduction pathways that are induced by IL-1 in these cells, and we have come to the following conclusions: Two functionally distinct IL-1R molecules are expressed, one linked to the protein kinase C (PKC) pathway and the other to the protein kinase A (PKA) pathway. While the former receptor molecule has not been biochemically defined, the latter is the p80 IL-1R that has been cloned and sequenced. Activation of the PKC pathway by IL-1 leads to c-fos expression, while the interaction of IL-1 with the p80 IL-1R leads to the activation of two separate pathway, one linked to a tyrosine kinase that leads to c-jun and c-myb expression, and the other linked to a G protein that activates the adenylate cyclase, resulting in c-myc expression. In addition, we have evidence of crosstalk between these various pathways, leading to up- and down-regulation of c-jun and c-fos.

D 222 REGULATION OF INTESTINAL ALKALINE PHOSPHATASE EXPRESSION AS A MARKER FOR TUMOR SUPPRESSION IN HELA X FIBROBLAST CELL HYBRIDS.
Kathryn M. Latham and Eric J. Stanbridge, Department of Microbiology and Molecular Genetics, University of California-Irvine, Irvine, CA 92717.
The aberrant expression of alkaline phosphatases in human cancer has been widely observed, yet the significance is not known to be functional or one of misregulated gene expression. We have found that expression of intestinal alkaline phosphatase (IAP) is exclusively associated with the tumorigenic phenotype of segregants derived from suppressed nontumorigenic, transformed HeLa x fibroblast (H/F) cell hybrids. Also, an accumulation of genetic evidence indicates the involvement a putative tumor suppressor locus in the co-regulation of both IAP and tumorigenic expression in the hybrids. To determine whether IAP expression represents a critical oncogenic event in the tumorigenic conversion of the H/F cell hybrids, the nontumorigenic H/F cell line, CGL1, was transfected with a β-actin promoter/IAP cDNA expression vector. CGL1 transfectants that expressed high levels of functional IAP on their cell surface were isolated and subcutaneously injected into athymic nude mice. After twelve weeks, no tumors were observed. *Therefore, expression of IAP in the suppressed H/F cell hybrid is not sufficient to promote tumorigenicity.* Thus, IAP would appear to be a specific marker of tumorigenicity rather than a critical oncogenic factor. At present, we are studying the transcriptional regulation of the differential IAP expression as a marker for tumor suppression in the H/F cell hybrids. The results of northern and nuclear run-on analyses, as well as transient transfection assays of IAP promoter-CAT fusion reporter plasmids, indicate that a negative regulator may be acting at the level of transcription initiation. Experiments are in progress to determine whether the negative regulator of IAP transcription is also involved in suppression of tumorigenicity in the H/F cell hybrids.

D 223 PHOSPHORYLATION OF SERUM RESPONSE FACTOR AND DNA BINDING ACTIVITY,
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The serum response factor (SRF) has been expressed in insect cells (Sf9 cells) using the baculovirus system. The SRF expressed in this system has been purified to homogeneity by use of specific DNA affinity columns. This preparation of SRF has identical properties to HeLa SRF in terms of its DNA binding affinity, specificity and mobility on SDS PAGE gels. It can also activate in vitro transcription from a serum response element. The Sf9 SRF is phosphorylated and removal of phosphate results in a reduction in DNA binding affinity of about 10 fold. This binding can be restored by casein kinase II phosphorylation. Residues responsible for this effect are being mapped. Other post-translational modifications are also being investigated in this system.

D 224 EXPRESSION cDNA CLONING OF THE KERATINOCYTE GROWTH FACTOR RECEPTOR BY COMPLEMENTATION FOR AUTOCRINE TRANSFORMATION

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A novel expression cDNA cloning strategy was used to isolate the keratinocyte growth factor (KGF) receptor cDNA. The introduction of an appropriate expression cDNA library prepared from the mRNA of one cell type into another results in the forced expression of genes which might normally be silent in recipient cells. KGF is expressed in stromal fibroblasts and has the properties of a paracrine mediator of epithelial cell proliferation. Thus, we reasoned that introduction of an epithelial cell cDNA library into fibroblasts might create an autocrine loop by ectopic expression of the KGF receptor cDNA and result in morphological transformation of the recipient cells.

A mouse keratinocyte cDNA library was constructed in λpCEV27, a λ-plasmid composite vector designed for stable expression cloning. Transfection of NIH/3T3 fibroblasts with the library DNA induced several transformed foci. Among them, one transformant demonstrated the acquisition of specific high affinity binding sites for KGF. Several plasmids were rescued from the cells and one of them was found to have high-titered transforming activity. Structural analysis of the 4.2 kb cDNA insert of the plasmid revealed that it encoded a predicted membrane-spanning tyrosine kinase. Characterization of the KGF receptor expressed by the cloned cDNA will be described.

D 225 IMPAIRED SIGNAL TRANSDUCTION OF TWO COOH-TERMINALLY TRUNCATED GROWTH HORMONE RECEPTORS TRANSFECTED INTO RAT INSULINOMA CELLS.

Annette Møldrup, Nils Billestrup, Thomas Dyrberg, Jens Høiriis Nielsen, Hagedorn Research Laboratory, Niels Steensens Vej 6, DK-2820 Gentofte, Denmark.

Growth hormone (GH) stimulates proliferation and insulin production of normal rat β-cells and the pancreatic rat insulinoma cell line, RIN-5AH, through the action on specific receptors. Recently, we showed that transfection of RIN-5AH cells with a full length rat hepatic GH receptor cDNA ($GH\text{-}R_{1-638}$) fused to the human metallothionein promoter increases the receptor expression up to 6 fold and that the increased receptor expression is accompanied by a stoichiometric increase of GH-stimulated insulin biosynthesis. In order to identify domains in the GH-R of functional importance two COOH-terminally truncated GH-R's were constructed by site-directed mutagenisis. Codons for Lys-295 and Lys-455 were changed to termination codons leaving 5 and 214 amino acids after the putative transmembrane domain, respectively. Transfected clones selected for further analysis exhibited a 2-4 fold increase of basal GH-binding and a 7-12 fold increase of GH-binding when cultured in the presence of 50 μM Zn^{2+} compared with the parent cell line. No difference in affinity between the mutant and the endogenous receptors could be observed. By affinity cross-linking and biosynthetical labeling the relative molecular masses of $GH\text{-}R_{1-638}$, $GH\text{-}R_{1-294}$, and $GH\text{-}R_{1-454}$ were determined to 110 K, 49 K, and 80 K, respectively. Neither $GH\text{-}R_{1-294}$ nor $GH\text{-}R_{1-454}$ expression was associated with an increase of GH-stimulated insulin biosynthesis. We conclude that the COOH-terminal half of the cytoplasmic part of the GH-R is required for mediation of the insulinotropic effects of GH.

D 226 MODULATION OF INTRACELLULAR CALCIUM IN HUMAN DERMAL KERATINOCYTES BY GROWTH FACTORS AND HORMONES.

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The effect of growth factors and neuroendocrine hormones on intracellular calcium levels in human dermal keratinocytes was monitored using Fura-2 and dual wavelength spectrofluorometric techniques. Stimulation of the cells with bradykinin, thrombin, and lysophosphatidate increased cytoplasmic calcium. Epidermal growth factor was ineffective in mobilizing cytosolic calcium but blocked further responses of the cells to thrombin. Exposure of the cells to low extracellular calcium abolished the bradykinin but not the thrombin induced rise in intracellular calcium. We postulate that there are multiple signal transduction pathways in keratinocytes that contribute to calcium mobilization and subsequently differentiation. The role of these various signal transduction pathways in differentiation is being investigated.

D 227 CHARACTERIZATION OF ESSENTIAL AND NONESSENTIAL AUTOPHOSPHORYLATION SITES IN THE HUMAN INSULIN RECEPTOR,
Monica S. Murakami-Greene and Ora M. Rosen, Graduate Program in Molecular Biology, Cornell University Graduate School of Medical Sciences, New York, NY 10021.
Autophosphorylation of the insulin receptor (Ins R) on tyrosine residues has been proposed to activate the tyrosine kinase activity of the receptor, thereby playing a role in the transmission of insulin signals. To analyze this role of autophosphorylation, the major tyrosine autophosphorylation sites were mutated to phenylalanine by site directed mutagenesis. Two mutants with contrasting phenotypes are discussed here. The first has the tyrosines 1146,1150 and 1151 mutated (R-Y3), and the second has the two carboxy terminal tyrosines 1316 and 1322 mutated(C-Y2). These mutants were transfected into CHO cells and their responsiveness to insulin was compared to parental CHO cells expressing very few receptors or to cells expressing similar numbers of ATP binding site deficient mutant (Lys 1018 to Ala) or wild type human receptors. C-Y2 was equivalent to cells transfected with wild type Ins R for the five parameters measured: autophosphorylation, internalization, thymidine incorporation, 2-deoxyglucose uptake and endogenous substrate (pp185) phosphorylation. In contrast R-Y3 is unable to mediate these five insulin effects. This mutant, R-Y3, has anomalous autophosphorylation activity. The mutations, R-Y3 and C-Y2, did not alter insulin binding or receptor synthesis. In conclusion, the carboxy-terminal tyrosines are nonessential autophosphorylation sites for the parameters measured. The tyrosines 1146, 1150 and 1151, however, are essential for signal transmission.

D 228 PERMEABILIZED QUIESCENT FIBROBLASTS AS A NOVEL ASSAY SYSTEM FOR NUCLEAR FACTORS ACTIVATING CELLULAR IMMEDIATE EARLY GENES,
Sun-Yu Ng, Shu-Hui Liu and Andrew Yueh Yueh, Institute of Molecular Biology, Academia Sinica, NanKang, Taipei 11529, Taiwan, Republic of China.
The promoters of many cellular immediate early genes contain an essential DNA motif known as the Serum Response Element (SRE). At least three nuclear proteins, the 67 kD Serum Response Factor(SRF), the 62 kD Ternary Complex Factor(TCF) and the 62 kD Direct Binding Factor(DBF), interact with this DNA sequence. Since the induction of cellular immediate early genes in response to serum mitogenic signals can occur in the presence of protein synthesis inhibitors, this transcriptional activation process may involve protein phosphorylation/dephosphorylation of one or more of these three factors. We have developed an in vivo assay to assess the functional ability of nuclear factors to activate the transcription of cellular immediate early genes in permeabilized quiescent fibroblasts. Our initial experiments showed that HeLa nuclear extracts induced the transcription of the c-*fos* gene in serum-starved murine fibroblasts which were permeabilized by either physical (glass beads) or enzymatic (Streptolysin O) methods. HeLa crude extracts were fractionated in order to identify the active nuclear factors. SRF(p67) was purified by binding to a wheat germ agglutinin(WGA)-agarose column but the active factors remained in the WGA-unbound fractions.

D 229 INHIBITION OF V-*SRC*-INDUCED TRANSFORMATION BY GTPASE-ACTIVATING PROTEIN,
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Oncogenic transformation by pp60$^{v\text{-}src}$ appears to be dependent on p21^{ras}, as antibodies against p21^{ras} are able to reverse transformation by *src*. The activity of p21^{ras} itself is regulated by its cyclic association with GDP/GTP, where p21^{ras}-GTP is the active form and p21^{ras}-GDP is the inactive form. The GTPase Activating Protein (GAP) mediates the inactivation of p21^{ras} by facilitating the conversion of the active p21^{ras}-GTP to the inactive p21^{ras}-GDP. Recently, it has been demonstrated that GAP is tyrosyl-phosphorylated in pp60$^{v\text{-}src}$-transformed cells, suggesting a direct interaction between the two oncogenes.. Here we report that overexpression of GAP blocks transformation of NIH3T3 cells by pp60$^{v\text{-}src}$ and negatively regulates related signal transduction pathways. Overexpression of GAP does not, however, block transformation by v-*ras*. These results confirm the central role of p21^{ras} in the signalling pathway utilized by pp60$^{v\text{-}src}$.

D 230 **MACROPHAGE MARKERS IN BREAST ADENOCARCINOMA: SIGNAL FOR AN INVASIVE PHENOTYPE?**

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Previous results have shown an association between the presence of oncogene amplifications (*neu/int2*) and a marked immune cell infiltrate in breast cancer (1). A marked lympho-plasmocytic infiltrate has now been documented to be a poor pronostic factor in two major clinical series (2,3). We have characterized these infiltrating immune cells in 30 specimens of ductal infiltrating breast cancer using specific anti CD3 (T cells), anti CD68 (macrophages), anti CD57 (NK cells) and an anti pan-B cell antibody (L26). The majority of tumour infiltrating immune cells are T cells (40-50%) and macrophages (15-35%), whereas B cells and plasmocytes (10-20%) as well as NK cells (5%) are quantitatively less important. CSF-1 is a known specific macrophage chemo-attractant and growth factor and is normally secreted by monocytes, macrophages, endothelial and stromal cells. We have previously shown that this growth factor is also produced by *invasive* breast cancer but not by in-situ (*pre-invasive*) cancer. Thirty tumour samples of ductal infiltrating type from previously untreated breast cancer patients (18 with oncogene amplification, 12 without), 15 of lobular infiltrating and 14 from in situ carcinoma were analized for the expression of CSF-1 and *fms* (the receptor for CSF-1). The expression of both these proteins by normal glandular epithelium as well as by endothelium, stromal cells, macrophages and TIL's was also assessed. Primary antibodies were: to *fms* (monoclonal antibody 2E8); to CSF-1 (rabbit polyclonal antibody 52P4). Tumour cells showed a mosaic staining pattern with antibodies to CSF-1 in invasive carcinoma, with a very high expression in areas of maximal stromal invasion (90%) and absent or trace staining in pre-*invasive* carcinoma. Macrophages were equally intensively positive. Expression of *fms* is typically more uniform in distribution and more variable in intensity and present in 20/27 tumours with high CSF-1 expression. We never observed a concomitant expression of both CSF-1 and fms in in-situ carcinoma. We therefore postulate that the associated expression of both CSF-1 and its receptor are intimately linked to the invasive potential of breast adenocarcinoma. Moreover *Neu* amplification and high CSF-1 expression by the tumour appear to be linked (P=0.04 ; P Yates = 0,09). We suggest that CSF-1 is a key marker of tumour *invasive* behaviour and that the production of CSF-1 might be stimulated via the *c-erbB2/neu* signalling pathways; the presence of large amounts of macrophages being a secondary event. Alternatively, a cooperative interaction between macrophages and carcinoma cells, possibly via trans-acting regulatory elements, might be inducive in its expression. (1) R Tang, B Kacinski, S Scholl et al. J Cell Biochem 44:1,1990 (2) JM Kurtz, J Jacquemier, JM Spitalier et al. Cancer 65:1867,1990 (3) PP Rosen, S Groshen, S Hellman et al. J Clin Oncol 7:1239,1990.

D 231 PARTIAL PURIFICATION AND CHARACTERIZATION OF CYTOSOLIC PROTEIN TYROSINE KINASES FROM HUMAN BREAST CANCERS, A.E. Ottenhoff-Kalff, B.A. van Oirschot, G. Rijksen, G.E.J. Staal
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In recent years a number of oncogene products and growth factor receptors with protein tyrosine kinase (PTK) activity has been identified in breast tumors, some of which have possible relevance for prognosis and diagnosis. We have previously shown that the total PTK activity of cytosolic and membrane fractions of breast carcinomas is significantly higher as compared to that in benign breast disease and normal breast tissue. PTK activity was measured in a non radioactive dot-blot assay using the artificial substrate poly(glutaminic acid:tyrosine = 4:1) (PGT), enabling the assay of a large number of samples at once. (G. Rijksen et al., Anal. Biochem. 182:98, 1989) We now report partial purification of the PTK's from the cytosolic breast cancer fraction using hydrophobic interaction chromatography with a tyrosine agarose column (twice), followed by anion exchange chromatography on a Mono Q column (Pharmacia). The enzyme activity is recovered from the tyrosine agarose columns by applying a linear gradient of ammoniumsulfate, first downwards from 0.8 M to 0 M $(NH_4)_2SO_4$, then upwards from 0 M to 0.5 M $(NH_4)_2SO_4$. After these two steps, the PTK activity is fractionated on a Mono Q column using fast protein liquid chromatography. By chosing this purification step, we can show that there are at least two forms of PTK, designated type I and type II. It appears that there is a consistent difference of these two types of PTK activity in their behaviour towards both of the substrates, ATP and PGT. Also, using polyclonal antibodies against the EGF receptor and the neu oncogene product, these two forms cross-react in a different manner. Further purification of these two PTK forms will allow identification of the oncogene products and/or growth factor receptors involved.

D 232 Identification of the Regulatory Phosphorylation Sites in pp42, a Mitogen-Activated Protein Kinase, Anthony J. Rossomando[1], D. Michael Payne[1], Paul Martino[3], Alan K. Erickson[2], Jeffrey Shabanowitz[3], Donald F. Hunt[3], Michael J. Weber[1], and Thomas W. Sturgill[2], Departments of [1]Microbiology, [2]Internal Medicine, and Pharmacology, [3]Chemistry, University of Virginia, Charlottesville, VA 22908

MAP kinase, a 42 kDa cytosolic serine/threonine kinase, is activated within 5-10 min of addition of any of several peptide growth factors or phorbol esters to quiescent cells, by a mechansim requiring tyrosine and threonine phosphorylation of the enzyme. MAP kinase activity can be completely inactivated by treatment with either phosphatase 2A or CD45, accompanied by essentially complete removal of either threonine or tyrosine phosphate, respectively. Although the *in vivo* functions of MAP kinase remain to be established, evidence from several laboratories supports the view that MAP kinase functions in a kinase cascade resulting in activation of an S6 kinase of the 90 kDa rsk family and phosphorylation of ribosomal protein S6. Understanding the mechanism of activation and *in vivo* function of MAP kinase would be aided considerably by identification of the protein sequence containing the regulatory sites of phosphorylation. *In vitro*, MAP kinase phosphorylates microtubule-associated protein 2 (MAP-2), *Xenopus* S6 kinase II and myelin basic protein (MBP); however, the more commonly used substrates (e.g., histone, casein, etc.) are not phosphorylated. Following mitogen stimulation, multiple protein kinases are activated, including at least one closely related enzyme, pp44, which can be resolved from pp42 chromatographically. A similar enzyme has also been identified following stimulation of sea star *Pisaster ochraceus* oocyte maturation. Here, we report results of studies of the phosphorylation sites in murine MAP kinase, by peptide mapping and mass spectrometry. We find that both tyrosine and threonine regulatory sites reside in a single tryptic phosphopeptide. The amino acid sequence of this phosphopeptide was determined by a combination of tandem mass spectrometry, using electrospray ionization methodology, and Edman degradation.

D 233 RPK1, A NEW AND ESSENTIAL PROTEIN KINASE GENE IN *S. CEREVISIAE*

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More then a dozen genes coding for protein kinases have been identified in the yeast *Saccharomyces cerevisiae*. They often have a crucial role in a particular cellular mechanism like mitosis, mating or sugar assimilation, but only a very few of them are essential for vegetative growth. The latter must therefore have a pivotal and non-redundant role in the cell.

We have identified a novel protein kinase gene, named **RPK1**, which is located adjacent to the **ACT2** gene (coding for an actin-like protein) on chromosome IV. The 2,292 nt-long **RPK1** reading frame encodes a 764 amino acid polypeptide (Mr 86,768). This gene is transcribed in proliferating cells to produce a 2,600 nt-long poly-A^+ mRNA of very low abundance. Gene disruption experiments clearly demonstrated that the **RPK1** gene is essential for vegetative growth.

Computer analysis suggests a bipartite organization for the **Rpk1** protein, with a hydrophilic N-terminal domain (residues 1-420) and a C-terminal domain (residues 421-764) showing all the characteristic features of the catalytic domains of known protein kinases. Strikingly, the homologies found with other catalytic domains of protein kinases are rather low and not confined neither in one genus nor in one family of kinases. This indicates that **Rpk1** belongs to a new class of yeast protein kinases. More interestingly, the "regulatory" N-terminal domain displays some similarities with the rod domain from members of the intermediate filaments multigene family (vimentin, laminin, ..) suggesting a possible physical interaction with those components of the cytoskeleton.

Recent experiments imply the **Rpk1** protein kinase in the pheromone-induced signal transduction pathway. Nevertheless this kinase must also be part of another vital process to explain its absolute requirement for vegetative growth.

D 234 CLONING OF A SECOND RECEPTOR FOR INTERLEUKIN-1. J.E. Sims, C.J. McMahan, B. Mosley, J. Slack, S. Lupton, D. McKean,* D. Benjamin,+ M.K. Spriggs, D. Cosman, S.K. Dower. Dept. of Molecular Biology and Biochemistry, Immunex Corporation, Seattle, WA 98101. *Mayo Clinic, Rochester, MN. 55905. +University of Oklahoma, Oklahoma City, OK 73104.

Using an improved method of direct expression cloning in mammalian cells, we have isolated cDNAs encoding a novel IL-1 receptor (type II IL-1R) from human B cells. As in the type I IL-1R, the extracellular, ligand-binding portion of the type II IL-1R is comprised of three immunoglobulin-like domains, although the degree of amino acid sequence identity between the two IL-1 receptors in this segment is only 28%. In contrast to the type I receptor, which has a 213 amino acid cytoplasmic portion, the type II receptor has only 29 amino acids intracellularly. Northern blot analysis indicates that high levels of type II IL-1R mRNA exist not only in B lymphoblastoid lines, bone marrow and activated neutrophils and monocytes, but also in placenta, activated keratinocytes and activated T cells.

D 235 CHARACTERIZATION OF THE MURINE INTERLEUKIN-5 RECEPTOR : EVIDENCE FOR SUBUNIT-RELATIONSHIP WITH THE INTERLEUKIN-3 RECEPTOR, Tavernier Jan, Van der Heyden José, Plaetinck Geert and Devos René, Roche Research Gent, Plateaustraat 22, B-9000 Gent, Belgium.

The presence of interleukin-5 receptors (Il-5R) has been demonstrated on B cells and on eosinophils. We will present two lines of evidence suggesting that the Il-5R on B cells has a common or homologous subunit with the Il-3R.

1. Two types of monoclonal antibodies (mAbs) were derived from rats immunized with the Il-5 or Il-3 dependent mouse B13 pre-B cell line. A first set of antibodies recognizes a 50 kD membrane protein and completely blocks the binding of only Il-5 but not Il-3 to B13 cells. A second group immunoprecipitates a 130-140 kD doublet and partially blocks the binding of both Il-5 and Il-3 to the same cells.
2. Internal amino acid sequences were obtained from the immuno-affinity purified larger receptor subunit(s). All peptides are present in the published amino acid sequence of a component of the Il-3R (Itoh et ak. 1990 Science 247:324), and of a Il-3R-like protein (Gorman et al. 1990 Proc. Natl. Acad. Sci USA 87:5459).

Together, these data can be interpreted into a model in which ligand-specific chains associate with a common or closely related subunit. This might suggest an overlap, at the receptor level, of the signal transduction pathways by these two lymphokines.

D 237 FUNCTIONAL EXPRESSION OF A HAMSTER 5-HT2 RECEPTOR AND ANALYSIS OF ITS IMPLICATION IN CELL GROWTH CONTROL Ellen VAN OBBERGHEN-SCHILLING, Valérie VOURET, Jean-Claude CHAMBARD, Richard J. HASLAM* and Jacques POUYSSEGUR. *Centre de Biochimie, CNRS, Parc Valrose, 06034 Nice Cédex, France and *Department of Pathology, McMaster University, Hamilton, Ontario L8N 3Z5 Canada*
Serotonin (5-HT) is a multifunctional bioamine that exerts its biological effects *via* distinct membrane receptors, several of which belong to the structurally-related family of G-protein-coupled receptors. We have isolated a hamster fibroblast cDNA clone that encodes a serotoninergic receptor whose deduced amino acid sequence displays 94% identity with the rat brain 5-HT2 receptor. When expressed in *Xenopus* oocytes, the hamster receptor efficiently couples to the phosphoinositide second messenger system and leads to intracellular Ca^{++} mobilization in response to 5-HT. To determine the pharmacological properties of this receptor, and to evaluate the role of phospholipase C (PLC) activation in growth modulation by 5-HT, we have expressed it in hamster fibroblasts. Transfected cells that express 5-HT receptors coupled to PLC were selected using a novel method based on co-expression of the Na^+/H^+ antiporter gene. We show that: i) following co-transfection of the 5-HT receptor and Na^+/H^+ antiporter cDNAs in fibroblasts lacking antiporter activity (varients of the CCL39 line), 50% of the clones resistant to an acute acid load express functional receptors. The extent of 5-HT-stimulated PLC activation in independent clones correlates with their relative level of cRNA expression. ii) The pharmacological profile of the transfected receptor is consistent with it being of the 5-HT2 subtype. iii) 5-HT2 receptor expression leads to strong activation of PLC by 5-HT, without stimulating DNA synthesis. We conclude that activation of the PLC signalling pathway alone in these cells is not sufficient to trigger GO/G1 to S phase transition. The Na^+/H^+ antiporter gene provides an efficient selectable marker for expression of transfected cDNA sequences in cultured cells. Attempts to co-amplify the transfected genes are underway; the strategies involved and results of these experiments shall be discussed.

D 238 PARATHYROID HORMONE: TWO PATHWAYS TRIGGERED BY ONE MOLECULE, Edgar Wingender, Angela Hollnagel, Christian Duvos, Verónica Vargas and Hubert Mayer, Department of Genetics, Gesellschaft für Biotechnologische Forschung, Mascheroder Weg 1, D-3300 Braunschweig, Germany.

We have identified two functional domains within the parathyroid hormone (PTH) molecule: one (the N-terminal) domain acts, e.g., on alkaline phosphatase and ornithine decarboxylase activity via cAMP. The other domain which is located in the central part of the molecule, stimulates creatine kinase activity and cell proliferation and involves an increase in phospholipid turnover. The complex domain structure of PTH has been elucidated further by a series of engineered mutants. Using PTH-derived peptides and mutants, it can be shown that both domains trigger specific gene regulation mechanisms within the target cells of PTH such as osteoblasts. Our results further demonstrate that the N-terminal domain harbors the catabolic hormonal activities leading to bone resorption, while the mid-regional domain exerts anabolic effects on bone and cartilage cells and tissue. We also show that there is a mutual functional interaction between both domains and we will discuss the factors/ conditions that help to decide which domain is predominantly active.

D 239 REGULATION OF NUCLEAR PROTO-ONCOGENES BY A PROTEIN-SERINE KINASE HOMOLOGOUS TO A DROSOPHILA SEGMENT POLARITY GENE, Jim Woodgett, Ken Hughes and Bernd Pulverer, Ludwig Institute for Cancer Research, 91, Riding House Street, London W1P 8BT, UK.
We have been studying the posttranslational regulation of several nuclear proto-oncogenes such as c-*jun*, c-*myb* and c-*myc* by protein phosphorylation as a potential means for extracellular stimuli to influence gene transcription. The three aforementioned proteins are phosphorylated *in vitro* at physiologically relevant sites by a protein-serine kinase termed glycogen synthase kinase-3 (GSK-3). In the case of c-*jun*, phosphorylation negatively regulates DNA binding, an effect reversed by dephosphorylation in cells upon stimulation. We have recently isolated cDNA clones for this enzyme and found it to exist in at least two forms, derived from distinct genes, termed GSK-3α and GSK-3β. To assess the function of these proteins, both have been expressed in insect cells using a baculovirus vector and large amounts of each protein purified to homogeneity. Recent cloning of the *Drosophila* segment polarity gene, *zeste-white3*, has revealed a striking amino acid identity with rat GSK-3β protein, strongly suggesting that GSK-3β is the mammalian homolog of *zeste-white3*. The implications of this identity and experiments performed to determine its significance will be presented.

Signalling by G Proteins: Large and Small

D 300 IDENTIFICATION AND PURIFICATION OF A PROTEIN WHICH AFFECTS GAP ACTIVITY IN VITRO,
Charles F. Albright^, Maja Vito^, Steven B. Kanner*, Albert B. Reynolds*, Ling Wu^, J. Thomas Parsons*, and Robert A. Weinberg^, ^Whitehead Institute for Biomedical Research, Cambridge, MA 02142 and *University of Virginia Health Sciences Center, Charlottesville, VA 22908
To better understand the role of ras p21 and GTPase Activating Protein (GAP) in signal transduction, we looked for activities which affect the ability of GAP to interact with ras p21. Cytoplasmic lysates from bovine brains were screened using GAP purified from baculovirus-infected cells and c-H-ras purified from E. coli cells. An activity was found which stimulated GAP activity sometimes and inhibited GAP activity on other occasions.
The partially purified protein was used to raise polyclonal sera in mice. The resulting sera recognize a 210kD tyrosine-phosphorylated protein and an 85kD protein in rat1/v-src cells. Both proteins appear to contain the epitope and the interrelationship of these proteins is unclear. In the process of generating monoclonal antibodies which react with tyrosine-phosphorylated proteins (P.N.A.S. 87:3328-3332, 1990), monoclonal antibodies against a 210kD protein were generated. When these antibodies were tested in rat1/v-src cells, they recognized 210kD and 85kD proteins. These appear to be the same proteins recognized by the polyclonal sera.
Using the monoclonal antibodies to follow the protein by Western blot, a 60kD fragment of the 85kD protein was purified from bovine brain. The resulting preparation contains an activity which inhibits GAP activity in vitro. We are trying to determine whether this activity is a property of the 60kD protein. Partial amino acid sequence is also being determined so the corresponding gene may be cloned.

D 301 SITE OF PHOSPHORYLATION OF RAP1B BY PROTEIN KINASE A.
Daniel L. Altschuler and Eduardo G. Lapetina, Division of Cell Biology, Burroughs Wellcome Co, Research Triangle Park,NC 27709.
A number of recent reports have demostrated the existence of small MW GTP-binding proteins in a wide variety of cell types. In platelets and HEL cells, some of these proteins are phosphorylated by agonists that increase cAMP levels. We previously showed that phosphorylation of a Ras-related protein causes its translocation from the membrane to the cytosol, suggesting its potential role in signal transduction mechanisms. Biochemical data from our laboratory demonstrated that this protein is Rap1B, and not Rap1A as previously suggested. To further characterize the phosphorylation site/s, we have cloned the Rap1B cDNA from a platelet library and made several mutations in the putative phosphorylation site/s, using a PCR approach. These clones were used for transfection in a transient expression system (COS cells), and the results showed that a serine next to the CAAX motif is phosphorylated by protein kinase A.

D 302 RECOMBINANT RAS PROTEINS AND GDP DEPHOSPHORYLATE UBIQUITOUS 17KD PHOSPHOPROTEIN,
Joseph M. Backer and Charmaine Mendola, Molecular Biology Section, Medical Research Division, Lederle Laboratories, American Cyanamid Company, Pearl River, NY 10965.
We have previously reported that recombinant ras proteins and guanine nucleotides modulate phosphorylation of the 17 and 36 kd rat liver mitochondria-associated proteins (PNAS,vol.83,pp6357-6361, 1986.). We now report that the 36kd protein detected in the SDS-PAGE is an unreduced dimer of the autophosphorylating 17kd protein. The 17kd protein is found both in the membrane and the soluble fractions of rat liver, as well as in various rodent and human cell lines. The 17kd protein utilizes both ATP and GTP as substrates in the autophosphorylation reaction. Free GDP or recombinant ras proteins purified as complexes with tightly bound GDP dephosphorylate the 17kd phosphoprotein. In both cases the phosphate group is transfered from the phosphoprotein to GDP. Ras protein monoclonal antibody Y13-259 inhibits ras protein induced dephosphorylation of the 17kd phosphoprotein and formation of GTP. Physiological significance of the 17kd protein and its relevance to the product of the recently described human metastasis supressor gene, NM23, is under investigation.

D 303 ANALYSIS OF A LINKAGE BETWEEN P21RAS AND THE INSULIN SIGNAL TRANSDUCTION PATHWAY. J.L. Bos, B.M.Th Burgering, R. Medema, A. Maassen, A.M.M. Smits, G.J. Pronk and F. McCormick [1]. Sylvius Laboratory, Leiden, The Netherlands. [1]Cetus Corporation, Emeryville, CA.
Using rat-1 cells overexpressing H-ras, we found evidence for a linkage between p21ras and the insulin/IGFI signal transduction pathway (1). We have studied various aspects of this p21ras function. First, we found that insulin did not induce c-fos and c-jun in untransformed- and in mutant ras-transformed cells, but did induce these genes in cells transformed by overexpression of normal p21ras. This indicates that overexpression of normal p21ras and not mutant p21ras provides a linkage between insulin receptor stimulation and gene induction. We are currently analyzing whether insulin can affect the GTP/GDP ratio of p21ras.Secondly, we investigated the role of protein kinase C (PK-C) in insulin-induced gene expression. The induction of c-fos could be inhibited by pretreating the cells with the phorbolester TPA, suggesting that a functional PK-C is necessary. The induction of c-jun, however, is not inhibited by TPA pretreatment. Thirdly, we have investigated the possibility that insulin induces the phosphorylation of GAP and its associated proteins, as recently found for EGF, PDGF and tyrosine kinase oncogenes. However, in NIH/3T3 cells overexpressing the insulin receptor 100-fold, insulin did not induce tyrosine-phosphorylation of GAP and its associated proteins. We conclude that p21Hras mediates the signal generated by insulin to induce gene expression.
(1) Burgering et al., 1989, Mol. Cell Biol. 9, 4312-4322;

D 304 THE NEUROFIBROMATOSIS TYPE 1 GENE SHARES SEQUENCE HOMOLOGY WITH IRA1 AND IRA2, TWO NEGATIVE REGULATORS OF THE RAS-cAMP PATHWAY. Arthur M. Buchberg, Luis F. Parada, Linda S. Cleveland, Nancy A. Jenkins, and Neal G. Copeland, ABL-BRP, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702.
Neurofibromatosis type 1 (NF1) is a very common human autosomal dominant disorder affecting 1/3500 individuals. The disease is characterized by abnormal proliferation of many tissues derived from the neural crest and patients with NF1 have an increased risk of developing various malignancies. The recent cloning of the NF1 gene now makes it possible to molecularly dissect the function of the NF1 gene and determine its role in control of cell proliferation. The autosomal mode of inheritance of the mutant NF1 gene combined with its frequent disruption in many patients with NF1 have strongly suggested that NF1 is encoded by a tumor suppressor gene. We have sequenced a portion of the murine NF1 gene and have shown that the predicted amino acid sequence is 99.2% identical with the corresponding region of the human NF1 gene. Northern analysis has identified NF1 transcripts in mouse tissues that are equivalent in size and complexity to those observed in human tissues. In situ analysis of mouse embryos are in progress and will be described. Finally, computer searches have identified amino acid homology between NF1 and IRA1 and IRA2, two genes identified in Saccharomyces cerevisciae that have homology to mammalian GAP and that function as negative regulators of the Ras-cAMP pathway. Collectively, these studies provide important new insights into the function of the NF1 gene. This work was supported by the National Cancer Institute, Department of Health and Human Services, under contract NO1-CO-74101 with ABL

D 305 **REGULATION OF THE GTPase ACTIVITY OF p25^{RAB3A}: EVIDENCE FOR A RAB3A GAP.** Ethan S. Burstein, Kimberly Linko, Zhijun Liu, and Ian G. Macara, Department of Biophysics, University of Rochester Medical Center, Rochester, New York 14620.
The rab3A gene product is a 25 kilodalton guanine-nucleotide binding protein, expressed at high levels in neural tissue, and which has about 30% homology to ras. Purified recombinant rab3A protein and p25^{rab3A} purified from bovine brain membranes have been used as substrates to look for factors that regulate its biochemical activity. A CHAPS-soluble factor associated with rat brain membranes exists that accelerates the GTPase activity of both mammalian and recombinant p25^{rab3A}. The activity was thermolabile, sensitive to trypsin, and behaved like an integral membrane protein. GAP activity towards p25^{rab3A} was also detected in the cytosolic fraction. This activity was observed in all other tissues examined, in addition to brain. Based upon dose-response data, the rab3A-GAP activity from rat brain was approximately equally distributed between cytosolic and membrane fractions; no activity was found in the nuclear fraction. Thus, the subcellular distribution of rab3A-GAP activity is similar to the subcellular distribution of p25^{rab3A}. The tissue distribution of rab3A-GAP activity appears to be ubiquitous, unlike the tissue distribution of p25^{rab3A}. Recombinant ras-specific GAP had no effect upon the GTPase activity of p25^{rab3A}. By gel filtration chromatography, the factor in rat brain cytosol has a molecular size between 300,000 and 400,000 daltons.

D 306 THYMOCYTE EMIGRATION AND MIGRATION DISRUPTED BY PERTUSSIS TOXIN,
Karen E. Chaffin, Chan R. Beals and Roger M. Perlmutter, Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195
Thymocytes (and mature lymphocytes) express high levels of the pertussis toxin-sensitive G_i proteins, G_{i2} and G_{i3}, which participate in as yet undetermined signal transduction pathways. To explore roles for G_i proteins in T cells, we expressed the catalytic S1 subunit of pertussis toxin in the thymocytes of transgenic mice using the lymphocyte-specific *lck* promoter. The S1 subunit is an ADP-ribosyltransferase that modifies, and inactivates, the α subunit of G_i proteins. Transgenic thymocytes contained active S1 protein and exhibited >90% depletion of unmodified (i.e. functional) G_i protein α subunits. This depletion of functional thymic G_i proteins did not detectably alter signal transduction via the antigen receptor, however mature T cells accumulated in transgenic thymuses and failed to populate peripheral sites. Mature, S1-containing thymic T cells transferred to the bloodstream of a syngeneic host remained there, whereas normal mature thymocytes and T cells migrated to peripheral lymph nodes. This migratory defect was not due to deficient expression of common adhesion/homing molecules, since mature transgenic thymocytes expressed cell-surface Mel-14, LFA-1, CD2, CD4, CD8 and CD3 at levels appropriate for mature, peripheral T cells. In addition, mature transgenic thymocytes performed normally in phorbol ester-induced aggregation and allo-MHC proliferation assays, demonstrating that LFA-1, the avidity of which can be regulated by T cell activation, functions normally in these cells. These experiments indicate that a novel PT-sensitive signal transduction pathway, probably involving a G_i protein, is required for T cell emigration from the thymus and the bloodstream.

D 307 ROLE OF G PROTEIN IN TRANSFORMATION AND EXPRESSION OF NATURAL ANTIBODY BINDING SITES
Donna A. Chow, David F, Tough, Wai Ching Tse and Paul A. Sandstrom, Department of Immunology, University of Manitoba, Winnipeg Manitoba, MB., Canada R3E OW3.
Infection of C3H 10T½ cells with the v-H-ras bearing raszip6 vector followed by selective growth in G418 produced populations of cells and clones which were transformed looking, tumorgenic and expressed the guanine nucleotide binding protein p21. These cells exhibited 1-2½ fold increases in serum natural antibody (NAb) binding measured by flow cytometry suggesting an NAb susceptible stage of tumor development. Furthermore, zinc induction of ras p21 expression in 2H1 produced a co-ordinated increase in their ability to bind NAb. In vivo IP administration of whole serum NAb on the two days prior to and just before the SC injection of 10^5 zinc-induced 2H1 cells into syngeneic C3H/HeN mice with $ZnSO_4$ in their drinking water decreased the early 30-day tumor frequency from 3/6 to 1/6 with mean latencies of 13 and 11 days respectively. In contrast, introduction of the tyrosine protein kinase oncogene src transformed 10T½ cells and produced small but consistent and significant decreases in their NAb binding capacity, demonstrating that the regulation of NAb binding sites is not dependent on transformation alone. In a different model, NAb binding by the murine T-cell lymphoma L5178Y-F9 increased upon exposure to the tumor promoter TPA and this was abrogated by the PKC inhibitor H7 suggesting that NAb binding is regulated through PKC. The data therefore suggest that although ras and src are considered to act along a common biochemical pathway in cell transformation, src may fail to stimulate a separate NAb binding pathway or may activate an additional pathway which leads to the down regulation of NAb binding capacity. (Supported by the NCI and MRC of Canada.)

D 308 PURIFICATION OF A 110 kDa CYTOSOLIC PLA_2 FROM RAW 264.7 CELLS, James D. Clark, Nina S. Milona, Chakkodabylu S. Ramesha* and John Knopf, Drug Discovery, Genetics Institute, Inc. Cambridge, MA 02140 , *Department of Inflammation Biology, Syntex Research, Palo Alto, CA 94303
Although there is accumulating evidence that PLA_2 activity is regulated by G-proteins and/or kinases, little is known about the cytosolic PLA_2 that interacts with these signal transduction systems. We have purified the major dithiothreitol-insensitive PLA_2 from the murine macrophage cell line RAW 264.7 cells by 30,000-fold using sequential chromatography on phenyl-5PW, Mono Q, HPHT, and G3000-SW columns. This new $cPLA_2$ is a105 kDa protein which selectively hydrolyzes fatty acids at the *sn*-2 position, and preferentially releases arachidonic acid when incubated with neutrophil membranes. Interestingly, a greater than 5-fold increase in PLA_2 activity is noted as the calcium concentration increases from the levels found in resting cells to those observed in activated cells. The identification of PLA_2 as a 105 kDa protein is consistent with our purification of a 110 kDa PLA_2 from the human cell line, U937. Recently, Leslie et al. (BBA, 963, 476, 1988) and Diez and Mong (JBC, 265, 14654, 1990) have attributed PLA_2 activity to a 60 kDa protein from the RAW 264.7 and U937 cell lines, respectively. However, the final specific activities reported by these two groups were 10 and 200 times lower, respectively, than the specific activities we have seen. Interestingly, in both of our purifications, a 60 kDa contaminant was one of the last proteins to be separated from the PLA_2 activity. This contaminant in RAW 264.7 cells has been shown by amino terminal sequencing to have 100% sequence identity to the murine 60 kDa polypeptide protein disulfide isomerase. Therefore, it is possible that the 60 kDa protein previously identified as PLA_2 is actually protein disulfide isomerase.

D 309 ***ras* p21 PROTEINS IN MEMBRANES OF REGENERATING RAT LIVER,**
Jennifer L. Cruise, Department of Biology, University of St. Thomas, St. Paul, MN 55105. A significant drop in p21 immunoreactivity occurs within the first 2 hr after two-thirds partial hepatectomy in the F344 rat, when the pan-reactive monoclonal antibody (mAb) Y13-259 is used for detection (*J. Cell. Physiol., (1989) 140: 195-201.*) Other pan-reactive and type-specific mAbs to *ras* proto-oncogene p21s have been used to further characterize this early prereplicative change. The loss of p21 immunoreactivity observed (at a time when *ras* mRNA synthesis appears to remain stable) may be due to translocation, degradation, modification, or association of p21 with other factors that mask antibody recognition sites. Where both Y13-259 (which recognizes a region near the 'effector' domain of p21) and the type-specific antibodies (which recognize unique C-terminal domains) demonstrate reduced binding, we may consider degradation, translocation, and/or major modification to be occurring. Where Y13-259 binding alone is reduced, association with other factors such as GAP may be occurring. Other patterns of immunoreactivity might indicate p21/receptor complex formation.

D 310 ACTIVATION OF $P21^{RAS}$ UPON STIMULATION OF THE ANTIGEN RECEPTOR IN T-LYMPHOCYTES,
Julian Downward, Jonathan D.Graves, Patricia H.Warne, Sydonia I.Rayter and Doreen A.Cantrell, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, England.
The *ras* proteins bind and hydrolyse GTP: p21.GTP is the active form of the molecule while p21.GDP is inactive. The physiological signals responsible for controlling the activation state of $p21^{ras}$ have long been sought. We have recently shown that in intact T lymphocytes the activation state of $p21^{ras}$, as determined by the amount of GTP bound to it relative to GDP, is under the control of the T-cell antigen receptor. Protein kinase C mediates this stimulation of $p21^{ras}$ which appears to occur through the suppression of GAP activity. An inhibitor of GAP activity is stimulated in T-cells upon treatment with protein kinase C activators. A permeabilised cell system has been used to study this activation of $p21^{ras}$ further: this shows that the rate of guanine nucleotide exchange on p21 in both lymphoblasts and fibroblasts is greatly stimulated relative to on pure p21 *in vitro*. The activation state of $p21^{ras}$ in both cell types appears to be controlled by a dynamic balance between two highly active and opposing proteins, GAP and a guanine nucleotide exchange factor.

D 311 PREFERENTIAL INHIBITION OF THE ONCOGENIC FORM OF RASH BY MUTATIONS IN THE GAP-BINDING/"EFFECTOR" DOMAIN
Larry A. Feig*, Jackson B. Gibbs# and Dennis Stacey@ *Department of Biochemistry, Tufts University Health Sciences Campus, Boston, MA 02111 #Department of Molecular Biology, Merck Sharp and Dohme Research Laboratories, West Point, PA 19486 @Department of Molecular Biology, Cleveland Clinic, Cleveland, OH 44106
The biological activity of oncogenic ras was reduced to a much greater extent than that of normal ras by either of two mutations, D33H/P34S or D38N. These mutations reduced both coupling to downstream effector molecules, as assessed by the ability of mammalian ras to stimulate yeast adenylate cyclase, and interactions with GAP (GTPase activating protein). Although the mutations in normal ras reduced downstream coupling activity, this appeared to be offset by decreased GAP binding since the latter was associated with a large increase in the percentage of ras bound to GTP in vivo. In contrast, the same mutations lead only to decreased coupling to downstream targets in oncogenic ras. This was because oncogenic ras was already primarily bound to GTP in vivo, due to its resistance to GAP's negative feedback activity. These results imply that preferential inactivation of the oncogenic form of ras in human tumors may be achieved by reagents designed to interfere with the GAP-binding/"effector" domain of ras proteins.

D 312 **p21RAS INVOLVEMENT IN T-LYMPHOCYTE ACTIVATION**
Nicholas W. Gale[1,2], Linda S. Graziadei[1,2] and Dafna Bar-Sagi[1], [1]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724 and [2]The Graduate Program in Cellular and Developmental Biology SUNY at Stony Brook, Stony Brook, NY 11794

We have been investigating the role of p21ras in T-lymphocyte activation. Activation of T-cells *in vivo* is thought to require a dual stimulation; the critical stimulus occurs via interaction of the T-cell antigen receptor complex with a specific antigen appropriately presented on the surface of accessory cells, and in assosiation with the major histocompatability complex. A costimulatory activity is thought to be provided by interleukin-1 and interleukin-6 or interaction with other cells such as macrophages. This activation initiates a program of proliferation and differentiation in which a battery of over one hundred known specific genes are transcriptionally activated over a period of several weeks. Included among the early markers of T-cell activation are the induction of the interleukin-2 (IL-2) and the IL-2 receptor (IL-2R) genes. T-cell activation can be partially mimicked *in vitro* using the pharmacological agents phytohemagglutinin, a mitogenic lectin, and phorbol esters such as phorbol-meristate-acetate, an activator of protein kinase C. Using this *in vitro* model system we have looked at the effects of transient overexpression of oncogenic p21ras proteins on the expression of IL-2 and the IL-2R in a human peripheral T-cell line, Jurkat. A potential role of p21ras in the signal transduction that ensues during T-cell activation will be discussed.

D 313 PROSTAGLANDINS ACTIVATE RAS-GAP, Jia-wen Han and Ian G. Macara, Environmental Health Sciences Center, Department of Biophysics, University of Rochester Medical Center, Rochester, NY 14642
The *ras*-specific GTPase activating protein (GAP) is an important signal transducing molecule that regulates p21*ras* by accelerating its slow intrinsic GTPase activity, which switches p21*ras* to the GDP-bound "off" state. GAP may additionally function downstream of p21*ras* as an effector protein. The mechanisms that control of GAP are therefore of considerable interest. Tyrosine kinase can phosphorylate GAP, and associated proteins, and may inactivate it. Certain lipids, particularly arachidonic acid and acidic phospholipids containing arachidonate, also potently inhibit the GTPase activity of GAP upon p21*ras*. However, no mechanism for the stimulation of GAP has been identified. We now report that the effect of recombinant *ras*-specific GAP on recombinant Ha-*ras* is significantly enhanced by certain prostaglandins. The effect is saturable and prostaglandin-specific, and appears to be competitive with the inhibitory action of arachidonic acid. Cyclo-oxygenase, which synthesizes prostaglandins from arachidonic acid, is an early-immediate gene product induced by growth factors and certain oncogenes (Han *et al.*, PNAS USA 87, 3373-3377, 1990). GAP may therefore serve to integrate opposing signals generated by mitogenic factors, so as to control precisely the state of activation of p21*ras*.

D 314 Role for a novel G-protein in the regulation of B lymphocyte proliferation, M.M. Harnett & G.G.B. Klaus, Division of Immunology, NIMR, London NW7 1AA, UK.
Surface immunoglobulin (sIgM and sIgD) receptors on B cells transduce signals by provoking the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate the intracellular second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG). We have recently demonstrated that sIgM and sIgD receptors on murine B cells are coupled to PIP_2 hydrolysis by a pertussis toxin-insensitive G-protein, G_p. We now report a role for a novel G-protein in the regulation of B cell proliferation. Using nonhydrolysable GTP analogues in reversibly-permeabilised B cell culture system, we have recently obtained evidence for a second site of G-protein regulation in sIg-activated B cells distal to the hydrolysis of PIP_2 and important in the control of antigen receptor-mediated B cell proliferation.

D 315 MUTATIONAL ANALYSIS OF Rap1/Krev PROTEIN; SENSITIVITY TO GTPASE ACTIVATING PROTEINS AND SUPPRESSION OF THE YEAST cdc24 BUDDING DEFECT

Heinz Haubruck, Paul Polakis, Peter McCabe, Leah Conroy, Robin Clark, Michael Innis and Frank McCormick, Dept of Molecular Biology, Cetus Corp., Emeryville, CA 94608

The rap1/krev-1 and ras gene products share 50% amino acid identity. However, expression of the genes in fibroblasts results in apparently opposing effects; ras causes transformation while rap1/krev1 reverts the ras-transformed phenotype. We have examined the functional consequences of substituting regions of $p21^{rap1/krev1}$ primary structure with the corresponding nonhomologous regions contained in $p21^{ras}$. These mutants, and three additional $p21^{rap1/krev1}$ mutants containing single amino acid substitutions, were tested for stimulaton by the purified GTPase Activating Proteins(GAPs) specific for $p21^{ras}$ and $p21^{rap1/krev1}$. We also tested the mutants for their ability to suppress the *Saccharomyces cerevesiae cdc24* temperature sensitive mutation; a defect known to be rescued by RSR1, the yeast homolog of mammalian rap1/krev1. The following $p21^{rap1/krev1}$ mutants containing the indicated $p21^{ras}$ residues or single residue substitutions were generated: rap11($p21^{21-31}$); rap12($p21^{45-54}$); rap13($p21^{70-76}$); rap14($p21^{79-83}$); rap17(val-12); rap18(ile-116); rap19($p21^{61-65}$); rap22(glu-63). Although it has been shown that regions surrounding the so called effector domain are important for suppressor activity as well as transformation in ras/rap chimeras the $p21^{rap1/krev1}$-specific GAP stimulated all of the mutants with the exception of rap 17 (val-12) which was, as expected, unresponsive. $p21^{ras}$-specific GAP was inactive towards wild-type and all mutant rap proteins with the exception of rap 19 which was stimulated at a level approximately 10-fold less than that observed with $p21^{ras}$. Wild-type rap1/krev1, rap12 and rap19 were as effective as *RSR1* in suppressing the *CDC 24* mutation. rap11 appeared to be a better suppressor than wildtype while raps17 and 18 had no effect. These results will be discussed in the context of the structural regions of $p21^{rap1/krev1}$ required for its proposed biological functions.

D 316 **Structure and Functional Analysis of Human D2 Dopamine Receptors.**

Gillian Hayes, Lisa A. Selbie and John Shine. Neurobiology Division, Garvan Institute of Medical Research, St. Vincents Hospital, Darlinghurst, Australia, 2010.

Dopamine, an important neurotransmitter in the central nervous system, exerts its physiological effects through two pharmacologically distinct, D1 and D2, G protein-coupled receptors. Dopamine activation of D2 receptors is known to both inhibit adenylyl cyclase and to activate K^+ channels in vivo suggesting that multiple D2 receptor subtypes may be required to interact with different intracellular signalling pathways. We have shown by molecular cloning and polymerase chain reaction (PCR) analysis that at least two structural forms of the D2 receptor exist. These D2 receptor subtypes arise from alternate splicing of a single D2 receptor gene and differ by the insertion of 29 amino acids in the putative third cytoplasmic loop. As this region of the molecule is thought to determine which intracellular signalling mechanisms the receptor couples to, we are examining whether these structural subtypes represent functionally distinct forms of the receptor. To this end, the genes encoding the "short", D2B, and "long", D2A, receptor subtypes have been expressed in mammalian cell lines. Ligand binding and cAMP responses have been assayed in these cell lines to measure the qualitative or quantitative differences in the coupling of these subtypes to intracellular second messenger systems. The D2A receptor, expressed in Chinese Hamster Ovary (CHO K1) cells, displays the appropriate affinity for a range of D2-selective agonists and antagonists. Dopamine inhibition of forskolin stimulated cAMP production is also observed, consistent with the receptor coupling to Gi in this cell line. Various hybrid (e.g. D2 - Leutenising hormone receptor)and mutant D2 receptors have also been constructed in order to determine which regions of the receptor molecule are important for coupling to specific G-proteins and hence to different signalling pathways.

D 317 DETECTION OF ACTIVATED N-RAS ONCOGENES IN C57BL/6 X C3H/He MOUSE LIVER TUMORS INDUCED BY CIPROFIBRATE A POTENT PEROXISOME PROLIFERATOR,

Monika E. Hegi[1], Tony R. Fox[2], Steven A. Belinsky[1] and Marshall W. Anderson[1]. [1]Lab. of Mol. Toxic., NIEHS, Research Triangle Park, NC 27709 and [2]Dow Chemical Company, Midland, MI 48674

Peroxisome proliferators which are nongenotoxic in short term mutagenicity tests are hepatocarcinogens in rodents. The mechanism(s) by which these agents induce tumors is not known. A previous study by Tony R. Fox detected a lower frequency of activated H-ras oncogenes in mouse liver tumors induced by ciprofibrate than in spontaneous liver tumors (21% vs. 78%). Nine tumors which were negative for H-ras activation were evaluated for the presence of other transforming genes by the nude mouse tumorigenicity assay. Four of the tumors were positive in this assay and three contained an activated N-ras gene based on Southern blot analysis (identification of mutations in progress). At present no activated N-ras genes have been detected in spontaneous liver tumors. Differences in the frequency of H-ras activation in ciprofibrate induced vs. spontaneous tumors implies that ciprofibrate is not acting via promotion alone. The activation of N-ras suggests that this hepatocarcinogen may act also as an indirect genotoxic agent possibly via generation of oxygen radicals.

D 318 cDNA CLONING AND SEQUENCING OF A MEMBER OF *RHO* GENE FAMILY FROM BOVINE KIDNEY,

Keith A. Hruska, Ajay Gupta, Puran Bora, John R. Didsbury, and Ralph Snyderman, Jewish Hospital and Washington University, St. Louis, MO 63110 and Duke University, Durham, N.C. 27710

We have previously reported that bovine kidney cortical microsomes are ~ 50 fold enriched (c.f. bovine brain) in a low M_r GTP binding protein which is a substrate for botulinum C3 ADP ribosyltransferase (C3) activity. The best known C3 substrate is the *rho* gene product, a member of the *ras* superfamily. The possibility that the C3 substrate in bovine kidney is *rho* was investigated by screening a λgt10 bovine kidney cDNA library using a human *rho* A cDNA probe (Yeramian et al, Nucl. Acids Res. 15, p. 1869, 1987). Five cDNA clones of different sizes were identified. The largest insert (clone no. 6, 1.4Kb) hybridized with the other 4 clones, and all of these clones hybridized with *rho* A cDNA probe under high stringency conditions. This suggests that all the clones isolated were identical to *rho* A. Clone 6 was subcloned into pBluescript II and sequenced by the dideoxy chain termination method. The open reading frame consists of 579 nucleotides encoding a polypeptide of 193 amino acid residues (calculated M_r 21,770 Da) which is 91% identical with that of the Aplysia *rho* gene and completely identical with that of human *rho* A. Thus, we conclude that the low molecular weight (M_r 24kDa) GTP binding protein, a substrate for botulinum C3 ADP ribosyltransferase activity, present in bovine kidney is most likely a *rho* gene product. Future work will center upon eukaryotic expression of these clones and studying function of the *rho* gene product in the bovine kidney.

D 319 LITHIUM-STIMULATED PROLIFERATION IN CULTURED NORMAL MAMMARY EPITHELIAL CELLS IS ASSOCIATED WITH A SUSTAINED ELEVATION IN INOSITOL PHOSPHATES

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Lithium (Li^+), at an optimum concentration of 5-10mM, stimulates the proliferation of normal mouse mammary epithelial cells (MEC) in primary, serum-free, collagen gel culture alone and, more prominently, in synergism with growth factors and cyclic AMP. The effect of Li^+ on phosphatidylinositol (PI) turnover was characterized in MEC cultured in serum-free medium and labelled to steady-state with ^{3}H-inositol. Li^+ treatment (5 to 50mM) caused a sustained (hours) and concentration-dependent (8 fold at 50mM) accumulation of predominantly inositol monophosphate (IP). This effect was not due to changes in medium osmolarity and was not dependent upon any exogenous growth factors or insulin. Maximum IP accumulation was not achieved at 50mM although Li^+ is growth-inhibitory at prolonged exposure above 10-15mM. Inositol penta-and hexakisphosphates were also detected but were not modulated by Li^+. In cells prelabelled with ^{3}H-inositol or $^{32}PO_4$, Li^+ (15-30mM) treatment produced a detectable decrease of phosphatidylinositolmonophosphate among the inositol phospholipids. An effect of Li^+ (5 to 30mM) on the synthesis of inositol phospholipids was not detected as assessed by $^{32}PO_4$ incorporation; apparent inhibition at Li^+ exceeding 5mM was accompanied by a proportional decrease in radioactivity in the cellular aqueous compartment. Li^+ may elevate IP levels by inhibition of inositol-1-phosphatase activity and the stimulation of phospholipase C activity. The pertussis toxin sensitivity of the Li^+ effect as well as the G-protein mediated involvement of Li^+ on inositol phospholipid - specific phospholipase C activity are being examined. These results suggest that Li^+ may activate and potentiate PI-dependent signal transduction events leading to growth stimulation alone and in synergism with other signal transduction pathways.

D 320 **ANALYSIS OF PHOSPHOLIPASE A_2 EXPRESSION IN NORMAL AND TRANSFORMED FIBROBLASTS USING THE POLYMERASE CHAIN REACTION.**

Gordon A. Jamieson, Jr. and Ming Yu, Division of Molecular Toxicology - Department of Environmental Health, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Previously we determined oncogenic transformation to alter the ability of bradykinin and serum to stimulate PLase A_2 in cultured human and rodent fibroblasts. In the current study we wished to determine whether these changes were the result of altered PLase A_2 expression. The polymerase chain reaction (PCR) was used to determine which of the Ca-dependent PLase A_2 isotypes are expressed in normal and transformed fibroblasts. Total RNA, isolated from four cell types, was reverse transcribed (RT) and the resultant ss cDNA used as PCR template. Anchored PCR was performed using a degenerate primer corresponding to the Ca-binding region of the Ca-dependent PLase A_2s. Parallel PCR reactions using a B-actin primer controlled for differences in RT efficiency. PCR products of the predicted sizes were obtained for "pancreatic" (ca. 415bps) and "non-pancreatic" (ca. 595 bps) PLase A_2s. Rodent fibroblasts expressed the pancreatic form of PLase A_2, while human fibroblasts expressed both the pancreatic and non-pancreatic isotypes of PLase A_2. K-ras transformed NIH/3T3 cells expressed 2-4 fold higher levels of PLase A_2 than their non-transformed counterparts, while no difference in PLase A_2 expression was observed between WI-38 and SV40/WI-38 cells. Current studies are focused on determining whether the expression of Plase A_2 isotype(s) and the bradykinin receptor are coordinately regulated in normal and transformed cells.

D 321 INHIBITION OF ras-INDUCED GERMINAL VESICLE BREAKDOWN IN XENOPUS OOCYTES BY rap-1B, Eduardo G. Lapetina, Michael J. Campa, Luis Molina y Vedia, Bryan R. Reep, and Kwen-Jen Chang, Department of Cell Biology, Burroughs Wellcome Co., Research Triangle Park, NC 27709
In 1989 Kitayama et al. identified a cDNA clone (Krev-1) possessing the ability to reverse the transformed phenotype when introduced into a K-ras-transformed NIH/3T3 cell line. The Krev-1 protein, which is also recognized as rap-1A, was found to be approximately 50% identical to the ras proteins. Recently, rap-1A was shown to block the interaction of ras and GAP in vitro, fueling speculation as to its role in vivo. A closely related protein, rap-1B, has also been identified in platelets, HEL cells, neutrophils, and aortic smooth muscle cells. Although it is not yet known if rap-1B has anti-oncogenic activity, Lapetina et al. (1989) have shown the protein to undergo a phosphorylation-dependent translocation from the plasma membrane to the cytosol. We sought to investigate the effect of microinjected rap-1B on H-ras(val12)-induced GVBD in oocytes. Equimolar concentrations of rap-1B were found to totally block GVBD triggered by the oncogenic ras protein. However, in the presence of IGF-1, this inhibition was not observed. Rap-1B was also shown to be phosphorylated in oocytes, a modification which may modulate the activity of the protein in vivo.

D 322 **Post-translational Modification of the p25^{rab3A} protein,** Kimberley A. Linko-Stentz, Zhijun Lu, and Ian G. Macara, Department of Biophysics, University of Rochester Medical Center, Rochester, New York 14642.
The p25^{rab3A} protein is a small ras-related G protein which has been shown to be expressed only in secretory tissue such as neurons and to be localized to synaptic vesicles, presynaptic plasma membranes, and presynaptic cytosol at the subcellular level. The p25^{rab3A} protein is partially cytosolic; association with membranes is presumed to be mediated by post-translational modification of the protein since purified recombinant and mammalian p25^{rab3A} partition into the aqueous and detergent phases, respectively, in the Triton X-114 assay. In order to investigate these putative modifications, an immune serum which specifically recognizes the p25^{rab3A} protein in its native and denatured forms has been generated. Using this antiserum, modification of the protein by carboxyl-methylation has been detected. Preliminary data indicate that the protein is not palmitoylated. Data regarding these and other investigations of post-translational modifications as well as data illustrating the specificity of the antibody will be presented.

D 323 A GTPase-DEFICIENT Gα_{i2} MUTANT ATTENUATES RECEPTOR STIMULATED PHOSPHOLIPASE A_2 ACTIVITY, Joseph M. Lowndes, Sunil K. Gupta, Shoji Osawa, and Gary L. Johnson, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206
G proteins in an activated, GTP-bound conformation regulate intracellular effector systems. The GTPase activity of the G protein α subunit functions as a timer to control the lifetime of the activated conformation. Here we report that the expression of a GTPase deficient mutant of the G_{i2} α subunit (α_{i2}Q205L) in Chinese hamster ovary (CHO) cells inhibits the stimulation of adenylyl cyclase by cholera toxin and attenuates the activation of phospholipase A_2 (PLA_2) by thrombin and purinergic receptors. The effect on stimulated adenylyl cyclase indicates that α_{i2}Q205L is a constitutively active Gα subunit, and supports the model that α_{i2} is a direct inhibitor of the enzyme. In CHO cells, hormone stimulation of PLA_2 activity requires both a functional α_{i2} and the presence of calcium. Cells which express α_{i2}Q205L are not deficient in α_{i2}-regulated PLA_2 activity; treatment of these cells with ionomycin, a calcium ionophore, stimulates the release of arachidonic acid. Furthermore, the inhibition of the PLA_2 response to purinergic agonist, which mobilized calcium from both extracellular space and internal stores, was less than the inhibition of the response to thrombin, which mobilized calcium only from internal stores. Our findings indicate that α_{i2}Q205L alters the calcium regulation of the G_{i2}-PLA_2 effector complex, and constitutively inhibits adenylyl cyclase.

D 324 CONSTRUCTION OF RETROVIRAL VECTORS FOR EXPRESSION OF MUTATED RAB3A, A RAS-LIKE, SMALL GTP-BINDING PROTEIN.
Zhijun Lu and Ian G. Macara, Environmental Health Sciences Center, Department of Biophysics, University of Rochester Medical Center, Rochester, NY 14642.

Rab3A is a member of the rab gene family of ras-like G-proteins. Rab3A has been found only in exocytotic tissues and is located in secretory vesicle membranes, suggesting that it may be involved in the control of agonist-stimulated secretion. To gain further insight into the function of rab3A, we have constructed a mutant, N134->I134, which by analogy with similar mutants in ras and SEC4 (a yeast GTP-binding protein involved in secretion) is expected to exhibit a dominant phenotype over the endogenous protein. The I134 mutant has been expressed in E. coli and shown to possess impaired GTP-binding. The mutant and the wild-type rab3A cDNAs have been cloned into retroviral vector pMV7, and these constructs are being used to infect 3T3 fibroblasts, PC12 cells and primary hippocampal neurones, to determine their effect on cell growth, morphology and on neurotransmitter secretion.

D 325 OVEREXPRESSION OF pp60$^{c\text{-}src}$ ENHANCES AGONIST-INDUCED ACTIVATION OF ADENYLYL CYCLASE. D.K. Luttrell, W.P. Hausdorff*, M.G. Caron*, R.J. Lefkowitz* and S.J. Parsons**. Glaxo Research Labs, RTP, NC 27709. *Depts. of Medicine and Cell Biology, HHMI, Duke Univ. Med. Center, Durham, NC 27710. **Dept. of Microbiology, UVA School of Med., Charlottesville, VA, 22908.

We have previously shown that in C3H10T1/2 murine fibroblasts which overexpress avian c-*src*, activation of the β-adrenergic receptor results in a 5-10 fold enhancement of intracellular cAMP accumulation relative to the amount induced in the parental line. In this study, we show that overexpression of either wild type c-*src*, or a kinase defective c-*src*, does not result in a detectable alteration in β receptor number or affinity. *In vitro* analysis of isoproterenol-induced adenylyl cyclase activity indicates that overexpression of wild type pp60$^{c\text{-}src}$, but not kinase inactive pp60$^{c\text{-}src}$, is associated with a three-fold increase in ligand potency and up to a two-fold increase in efficacy of isoproterenol on adenylyl cyclase activity. No difference in adenylyl cyclase activity was observed between control cells or cells overexpressing wild type pp60$^{c\text{-}src}$ following treatment with NaF, suggesting that overexpression of c-*src* affects the activation of adenylyl cyclase proximal to, or upstream of, G_s activation and may enhance coupling of the β-receptor to G_s. Cells overexpressing wild type c-src show a 50% reduction in the ability of forskolin to activate adenylyl cyclase compared to the levels of activity induced in both the control and kinase defective cell lines. Therefore, overexpression of wild type c-*src* may affect two distinct steps in the regulation of adenylyl cyclase, exerting a positive effect at the level of G_s activation and a negative effect on adenylyl cyclase itself. These results raise the possibility that pp60$^{c\text{-}src}$ may phosphorylate one or more components involved in receptor-mediated activation of adenylyl cyclase.

D 326 INTERACTIONS BETWEEN p21^{ras} GTPase ACTIVATING PROTEIN (GAP), RECEPTOR TYROSINE KINASES, AND THEIR SUBSTRATES REQUIRES SH2 DOMAINS AND PHOSPHORYLATION. Mike Moran*, Christine Ellis, Anne Koch, Debbie Anderson, & Tony Pawson. Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto. *Present address: Banting & Best Department of Medical Research, University of Toronto, 112 College Street, Toronto, Ontario M5G 1L6, CANADA.

The p21^{ras} GTPase activity protein (GAP) is found predominantly as a monomer in the cytosol of normal cells. However, in cells expressing activated cytoplasmic or receptor protein-tyrosine kinases, GAP becomes phosphorylated on tyrosine and serine and forms distinct complexes with phosphoproteins including autophosphorylated receptors and proteins of M_r 62 kDa and 190 kDa (p62 and p190). v-*src* transformation induces approximately 8% of GAP to associate with the membrane, where it becomes phosphorylated on tyrosine and associates with highly tyrosine phosphorylated p62, which is also found in the cytosol. In contrast, the majority of GAP enters a distinct complex with p190 which is exclusively cytosolic and contains predominantly phosphoserine. The GAP-p190 complex is dependent on phosphorylation, and shows reduced GAP activity. EGF stimulation also induces a marked conversion of monomeric GAP to higher molecular weight species in rat fibroblasts. The interaction of GAP with ligand-stimulated tyrosine kinase receptors and p62 apparently reflects the affinity of GAP SH2 domains for proteins containing phosphotyrosine. These interactions have been reconstituted *in vitro* by using recombinant GAP SH2 domains. These results suggest that tyrosine phosphorylation induces GAP to form heteromeric SH2-mediated complexes, which are compelling candidates for regulators or targets of p21^{ras}.

D 327 H-ras FUNCTION IS ALTERED BY TREATMENT WITH THE PHORBOL ESTER, PMA, Suzanne K. Murphy, and David A. Prussack, Department of Biological Sciences, Philadelphia College of Pharmacy and Science, Philadelphia, PA 19104

We are employing an NIH3T3 cell line transfected by the H-ras oncogene under the control of a steroid inducible promotor to study the early effects of H-ras expression. In the presence of dexamethasone, cells exhibit an increase in phosphoinositide (PI) turnover, growth rate and saturation density as well as a morphology characteristic of the transformed phenotype. Concomitant with ras gene expression, levels of polyphosphoinositides decrease and inositol phosphate levels increase. PI turnover remains elevated following maximal expression of the ras product. We have found that the phorbol ester, phorbol 12-myristate 13-acetate (PMA), a potent tumor promoter and activator of protein kinase C, amplifies, then overrides, the effects of ras gene expression on PI turnover. During maximal ras expression, addition of PMA results in a rapid, marked (80%) stimulus of PI turnover followed by a gradual decrease towards control levels. Cell growth is also affected by PMA. In the presence of phorbol ester, cell growth occurs more slowly than in either control or dexamethasone treated cells. These results may indicate that in the system under study, changes in PI turnover and/or PKC activation are involved in aspects of cellular metabolism not necessarily related to growth control.

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D 328 PURIFICATION AND CHARACTERIZATION OF PI-PLC FROM HUMAN T LYMPHOCYTES, Joanne C. Pratt and Glen N. Gaulton, University of Pennsylvania School of Medicine, Philadelphia, PA, 19104

Stimulation of T lymphocytes by antigen or mitogen generates a complex series of molecular events that ultimately result in cellular proliferation. One early consequence of stimulation is the hydrolysis of membrane phosphoinositides by phosphatidylinositol specific phospholipase(s) C (PI-PLC). Purification of PI-PLC from human T lymphocytes was achieved using standard column chromatography. Two forms of PI-PLC were resolved by anion exchange. One of these forms, PI-PLC II, was purified by affinity chromatography and hydrophobic interaction chromatography to apparent homogeneity. This protein migrated as a single band of 68,000 under reducing conditions and as two bands of 58,000 and 116,000 under non-reducing conditions on a polyacrylamide gel. Cleavage of PI by purified PI-PLC showed strict pH, detergent, and calcium dependence. Preferential cleavage of PIP_2 over PI occurred at uM concentrations of calcium and PIP_2 hydrolysis was calcium independent. Antibodies to PLCa or human platelet PI-PLC did not recognize any proteins in human T cell cytosol, suggesting that T cells contain unique forms of PI-PLC.

D 329 **FcγR III (CD16)-OPERATED SIGNALS REGULATE ITS EXPRESSION ON NK CELLS,** Antonio Procopio, Cristina Galli, Rossella Paolini, Angela Santoni, and Luigi Frati, Depts. of Experimental Medicine, University of L'Aquila, L'Aquila 67100, and University La Sapienza, Rome, Italy, 00161.

This study demonstrates that FcγRIII-operated signal transduction, is mediated by G-proteins, and induces IP_3 generation and PK-C activation, and that, in turn, PK-C down-modulates CD16 expression on NK cells. In addition the cross-linking of CD16 by moAb or Ab-coated target cells, GTPγS induced IP_3 release in permeabilized NK cells and their membranes. Conversely, GTPβS inhibited IP_3 generation. *Vibrio cholerae* toxin (Ctx) strongly inhibited IP_3 generation and ADCC, while Ctx B subunit alone was not active. PK-C activation, induced by TPA or OAG, inhibited ADCC and induced the rapid disappearance of CD16 on NK cells. Staurosporin and H7, reversed the OAG and TPA-mediated effects. Most of the CD16 molecules were shedded. The remaining ammount of CD16 molecules were internalized.

Thus, our results suggest that a negative regulatory feed-back of FcγRIII-mediated functions exist thorough PK-C-dependent regulation of CD16 expression.

D 330 **ISOLATION OF MOLECULAR PROBES FOR THE BRADYKININ RECEPTOR USING THE POLYMERASE CHAIN REACTION.** Jennifer B. Richard and Gordon A. Jamieson, Jr., Division of Molecular Toxicology - Department of Environmental Health, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267
In order to obtain a better understanding of the processes which regulate cellular responses to the peptide bradykinin we have undertaken the isolation of a cDNA clone for the bradykinin receptor (BKR). We and others have reported the coupling of the BKR to its target effector enzyme(s) to be dependent upon a GTP-binding protein. As such the BKR can be expected to be a member of the superfamily of G-protein coupled receptors (GCRs). To date all GCRs characterized possess 7 putative transmembrane domains. Based upon these structural homologies Libert et al. (Science 244:569) synthesized degenerate oligonucleotides and with reverse-transcribed (RT) mRNA acting as template used the polymerase chain reaction (PCR) to isolate probes for several novel GCRs. Using a similar approach we have performed RT-PCR on mRNA isolated from cells expressing a high level of bradykinin receptors (K-ras transformed NIH/3T3 cells) and obtained two amplified oligonucleotide bands estimated to be 450 and 500 bps in length. These PCR products have been subcloned and partially sequenced. DNA sequence information obtained to date suggests the amplified PCR bands are derived from a novel GCR. Current work is aimed at isolating and characterizing a complete BKR cDNA and using a Xenopus oocyte expression system to determine whether these cDNAs encode the BKR.

D 331 MODULATION OF THE TRANSFORMING POTENTIAL OF H-ras BY MUTATION AT Phe 28, Michael H. Ricketts, Glenda Durrheim, Honor North, Marthinus J. van der Merwe, and *Arthur D. Levinson, US/MRC Centre for Molecular and Cellular Biology, P.O. Box 63, Tygerberg, 7505, South Africa, and *Genentech Inc., 460 Point San Bruno Blvd, South San Francisco, CA 94044.
We have constructed and analysed the effect of specific mutations on the transformation potential of p21(H-ras). Mutations in conserved amino-acids from position 25 to 34 were introduced into c-H-ras with a pre-existing activating mutation (valine-12). Conversion of amino-acid 28 from phenylalanine to aspartic acid (F28D) abrogated the transforming potential of the activated p21, while F28W effectively transformed Rat-1 cells. The inability of F28D to transform cells was associated with the absence of detectable GTP binding by the bacterially expressed protein. These results supplement X-ray diffraction studies which indicate an interaction between the aromatic rings of guanine and F28 of p21(H-ras). Expression vectors encoding c-H-ras without a pre-existing activating mutation (12G) were constructed with F28D, F28W and P34A. Of these F28W showed weak transforming activity in Rat-1 cells, and clones expressing this mutated p21 grew in soft agar. Different mutations at amino-acid 28 are therefore able to either up- or down-regulate transforming potential of p21(H-ras). The activation in F28W could be due to increased dissociation of GTP from p21 or to inhibition of GAP stimulation of GTP hydrolysis. Preliminary results indicate the former is operative.

D 332 **RAPID DESENSITIZATION IN BRADYKININ-STIMULATED INOSITOL MONOPHOSPHATE PRODUCTION: IMPLICATIONS FOR PEPTIDE ANALOG DESIGN,** Jan S. Rosenbaum & Dana Hance Wolsing, The Procter & Gamble Co., Corporate Research Division, Miami Valley Laboratories, P.O. Box 398707, Cincinnati, OH 45239-8707.
A 5 min pre-exposure of NG108-15 cells to 10^{-7}M bradykinin (BDK) is sufficient to induce a desensitization in subsequent BDK-stimulated IP production that is characterized by both a loss in BDK potency and a decrease in amplitude of response. Only the decrease in response amplitude is attenuated by down-regulation of protein kinase C (PKC) by prior long-term treatment of the cells with TPA, indicating an involvement of PKC activation in the desensitization process. [^{3}H]BDK binds to two distinct binding sites in NG108-15 cell membranes with 100-fold selectivity for the high affinity site (Site 1). The desensitization is accompanied by a loss of 67 ± 3% (N = 10) of Site 1; Site 2 and the K_d for BDK at both sites remain unchanged. Long term TPA treatment does not attenuate this receptor loss, indicating that the rapid desensitization involves both receptor-related and post-receptor mechanisms. We have also found that BDK antagonist analogs are ~10-fold less potent in antagonizing BDK-mediated IP production than would be predicted from their binding affinity at Site 1, a result which could be explained by the rapidly desensitizing property of the receptor. Hence, the desensitization can influence the determination of agonist and antagonist potency and may contribute to differences in analog behavior in different cells and tissues.

D 333 <u>IN VITRO</u> SYNTHESIS OF FUNCTIONAL G PROTEIN $\beta\gamma$ SUBUNITS, Carl J. Schmidt and Eva J. Neer, Cardiovascular Division, Brigham and Women's Hospital, Boston, MA 02115
The guanine nucleotide-binding proteins (G proteins), which play a central role in coupling membrane-bound receptors to intracellular effectors, are heterotrimers composed of α, β and γ subunits. The β and γ subunits form a functional monomer that does not appear to separate under physiological conditions. To characterize the individual subunits of $\beta\gamma$, the 36 kDa β subunit (β_1), brain γ (γ_2) and transducin γ (γ_t) were translated <u>in vitro</u> in a rabbit reticulocyte lysate system. Without γ subunits, the β subunits are not stable. We could not detect any interaction with α subunits when either β or γ were tested individually. Either co-translation of β and γ or mixing separately translated β and γ subunits yielded functional $\beta\gamma$ dimers that could be separated from monomeric β or γ. The sedimentation coefficient of <u>in vitro</u> translated $\beta\gamma$ (IVT$\beta\gamma$) increases from 3.4$\pm$0.1 S (n=4) to 4.3$\pm$0.1 S (n=4) when α_o and GDPβS was added, indicating that IVT$\beta\gamma$ could form a heterotrimer. This heterotrimer dissociates with GTPγS. Comparison of their partial specific volumes reveals that the <u>in vitro</u> products are less hydrophobic than the $\beta\gamma$ purified from brain. Tryptic digestion of IVT$\beta\gamma$ gave the same two products (27 kDa and 14 kDa) as purified $\beta\gamma$. In contrast, proteolysis of β without γ yielded only a 19 kDa product. Analysis of IVT$\beta\gamma$ subunits will provide ways to assess the function of these subunits and to determine the structural requirements for $\beta\gamma$ formation. Supported by NIH grant GM 36259 to EJN, and by the Charles A. King Trust, Boston, MA to CJS.

D 334 INTERACTION OF GUANINE NUCLEOTIDE RELEASING FACTOR (GRF) WITH P21ras.
V.M. Stathopoulos*, A. Wolfman*, D. R. Lowy+, A. Papageorge+, B.M. Willumsen# & I.G. Macara*. * Toxicology Dept., University of Rochester, Rochester, N.Y. 14642, + National Institutes of Health, NCI, Bethesda, MD 20892 & # Univ. Institute of Microbiology, Copenhagen Denmark.

The <u>ras</u>-proto-oncogene family codes for several small G proteins which bind guanine nucleotides and have slow intrinsic GTPase activities. The release of guanine nucleotides from the <u>ras</u> proteins is very slow in the presence of magnesium. A factor which specifically accelerates the release of GDP and GTP from <u>ras</u> has been found in rat brain cytosol (Wolfman & Macara, Science 248:67-69, 1990.). This factor, guanine nucleotide releasing factor (GRF), by catalyzing GDP/GTP exchange could function as an "on-switch" for <u>ras.</u>
We have used mutant <u>ras</u> proteins generated by linker-insertion-deletion mutagenesis to try and determine the binding domain on <u>ras</u> of GRF. Elucidation of the mechanism of interaction of GRF with <u>ras</u> is essential to an understanding of the <u>ras</u> oncogene protein in signal transduction.
GRF activity was assayed as described previously (Wolfman & Macara) using rat brain cytosolic extracts as a source of GRF, and ^{32}P-GDP-loaded <u>ras</u> proteins. GRF is specific for <u>ras</u> since no GRF activity was detectable using other small G-proteins (rab2, rab3A, rap1A). Preliminary results with deletion/insertions mutants of <u>ras</u> rule out GRF interaction with the effector domain, carboxy terminus and several other regions within the protein.
By comparing the homologies of the small G-proteins with <u>ras</u>, together with the mutant data, we speculate that the possible regions of interaction of p21<u>ras</u> with GRF include residues 20-30, 80-85 and/or 145-150. Several new mutant proteins containing deletions in these regions and ras/rap chimeras are presently being tested for responsiveness to GRF. Results from these and the previous experiments will be presented.

D 335 DESENSITIZATION OF THE CA^{2+}-MOBILIZING SYSTEM TO BOMBESIN BY HA-RAS, Florian Überall, Hermann Oberhuber, Karl Maly, Lars Demuth, Alexander Kiani, and Hans H. Grunicke, Department of Medical Chemistry and Biochemistry, University of Innsbruck, A-6020 Innsbruck, Austria.
Expression of the transforming human Ha-ras oncogene (val-gly mutation at codon 12) under the control of the mouse mammary tumor virus long terminal repeat (MMTV-LTR) leads to a rapid desensitization of the bombesin and serum growth factor (GF) induced intracellular Ca^{2+}-mobilizing system and inositol 1,4,5-trisphosphate (IP_3) formation. Half maximal depression of the $[Ca^{2+}]_i$ release is observed two hours after induction of $p21^{ras}$ and shows a maximum after six hours. The rapid desensitization of the Ca^{2+}-releasing system by Ha-ras is not caused by a down modulation or uncoupling of phospholipase C from corresponding bombesin receptors. A depletion of $[Ca^{2+}]_i$ stores by Ha-ras is unlikely as (i) the Ha-ras induced GF-independent stimulation of inositolphosphate formation occurs several hours after the reduction of the Ca^{2+} response and (ii) the loading rate of nonmitochondrial $[Ca^{2+}]_i$ stores was found to be unaffected. It is demonstrated that in permeabilized cells the expression of Ha-ras reduces IP_3-mediated release of intracellular Ca^{2+}. The desensitization of the Ca^{2+} mobilizing system could be mediated by a partial inhibition of IP_3-regulated Ca^{2+}-channels.

D 336 POST-TRANSLATIONAL MODIFICATIONS AND FUNCTION OF THE RAS-LIKE PROTEIN, p23^{rab2}. Chungwen Wei and Ian G. Macara, Departments of Biochemistry and Biophysics, University of Rochester, Rochester, N.Y. 14642

Rab2, a member of the small GTP-binding protein family, has recently been localized to Golgi-like structures and been implicated in the exocytic pathway. Our preliminary data show that rab2 is distributed in both the cytosol and membrane fractions in rat brain, and this protein migrates slightly faster than recombinant rab2 in SDS-PAGE, suggesting that some sort of post-translational modifications occur. Our preliminary data also show rab2 is expressed in NIH3T3 cells. With the immunoprecipitating anti-rab2 antibody we have developed, we can detect whether and what kind of post-translational modifications occur in this cell line.

Feig has shown that an S17 → N17 mutation in H-v-ras generates a ras protein which is defective in GTP binding and exhibits a dominant transformation-suppressor phenotype. We have constructed a mutant rab2 in the corresponding position and have it expressed in E. coli. We have purified this recombinant mutant protein and also transfected the mutated rab2 cDNA into mammalian cells. The effects of this mutation on the biochemical and biological properties of rab2 are currently under investigation.

D 337 IMMUNOREACTIVITY OF ISOPRENYLATED RAP1B WITH RAP1-SPECIFIC ANTISERA, Deborah A. Winegar, Carol A.-Ohmstede, Lily Chu, Bryan Reep and Eduardo G. Lapetina, Division of Cell Biology, Burroughs Wellcome Co, Research Triangle Park, NC 27709.

Polyclonal antisera were generated against synthetic peptides corresponding to distinct regions of the rap1 protein sequences. A "rap1-common" antiserum, prepared against an 18 amino acid segment of the rap1a protein near the proposed GTP-binding region, reacted with both rap1a and rap1b recombinant proteins expressed in E. coli and with two low molecular weight GTP-binding proteins of 22 and 24 kDa in unstimulated human platelets. An antiserum raised against a carboxyl-terminal peptide of rap1b containing the proposed site of post-translational processing, reacted strongly with bacterial-expressed recombinant rap1b and with a 24 kDa GTP-binding protein in platelets, but not with recombinant rap1a or a 22 kDa platelet protein. The mobility of this rap1b immunoreactive protein coincided with that of bacterial-expressed rap1b and not with the faster migrating form of rap1b that incorporates radioactivity from [^{3}H]mevalonic acid in the insect/baculovirus system. This suggests that our rap1b-specific antiserum recognizes only one form of rap1b, that which has not undergone carboxyl-terminal post-translational processing.

D 338 CLONING OF THE HUMAN FORM OF PHOSPHOLIPASE C-γ: OVEREXPRESSION IN NIH-3T3 FIBROBLASTS LEADS TO MALIGNANT TRANSFORMATION, Albert Wong, Michael Y. Wong, Eileen Shore, Heather McDanel, Naomi Haas and Andrew Godwin, Department of Medical Oncology, Molecular Oncology Program, Fox Chase Cancer Center, Philadelphia, PA 19111

We have obtained a full length clone for PLC-γ from a human glial tumor xenograft called D320. Sequence analysis shows that in the coding region, the human form has ~90% nucleotide homology and ~95% amino acid similarity when compared to the rat and bovine forms. To study its role in transformation, the coding region was placed into an expression vector under the control of a viral LTR and cotransfected with a plasmid encoding the gene for resistance to G-418. Following analysis by Southern blotting for integration into genomic DNA and by Northern blotting and Western blotting for overexpression of the human PLC-γ cDNA, five G-418 resistant subclones were chosen for further analysis. When analyzed in cell growth assays, these cell lines exhibited doubling times that ranged from 22-25 h as compared to normal which had an average time of 27.2 h. Each clone also attained a higher cell density which ranged from 1.4-1.8x that of control. In soft agar cloning assays, control fibroblasts showed less than 1% cloning efficiency, while the human PLC-γ containing subclones gave efficiencies of between 6% to 22%. In a Western blot analysis using an antiphosphotyrosine antibody, the five lines revealed increased amounts of phosphotyrosine while control cells only showed moderate labelling when grown in low serum media. Finally, each line was tested for tumorigenicity in athymic mice. 1×10^6 cells from each line were injected subcutaneously into each mouse. Three of the lines showed tumors in all mice injected (5/5) which took approximately 17-22 days to develop and grew rapidly thereafter. Of the other two lines, tumors developed in 4/5 and 3/5 mice with a latency period of 4-5 weeks. We conclude that this human PLC-γ clone is capable of transforming NIH-3T3 fibroblasts as assayed by these criteria. We have recently cloned a cDNA from normal tissue and are currently in the process of determining if any activating point mutations have occurred in our glial tumor derived cDNA.

D 339 VIDEOMICROSCOPY REVEALS NEW ASPECTS OF $[CA^{2+}]_i$ TRANSIENTS INDUCED BY RECEPTORS COUPLED TO POLYPHOSPHOINOSITIDE HYDROLYSIS, Daniele Zacchetti, Fabio Grohovaz, Emilio Clementi, Jacopo Meldolesi and Guido Fumagalli, CNR Cytopharmacol., Dept Pharmacol., Inst. S. Raffaele, Univ of Milan, Italy.
Activation of membrane receptors coupled to PPI breakdown results in increase of $[Ca^{2+}]_i$ generated by release from intracellular stores and opening of cationic channels. While a large body of evidence correlates PPI breakdown with release of the ion from specific intracellular organelles, it has recently proposed that influx can be directly coupled to receptor activation, possibly through a G protein interaction. We have used Ca imaging of PC12 cells loaded with Fura 2 to study the occurrence in single cells of influx and release induced by bradykinin (BK), ATP and carbachol (CCh). The results in more than 200 cells demonstrated that cells responsive to a given agonist can be divided in three categories, depending on whether only influx or release or both processes are elicited. Frequency distributions in the three categories were similar for BK and ATP but differed for CCh, where the majority of the cells showed influx only. When the same cell was challenged in sequence with the three agonists, unique response patterns were often observed. In a clone of PC12 cells obtained from the original population the frequency distribution of the responses peaked in one of the three categories suggesting that heterogeneity to a given agonist was due to the presence of multiple clones in the original PC12 cell population. In the same clone the responses to CCh differed from those obtained with BK and ATP indicating that each receptor is differently equipped to activate $[Ca^{2+}]_i$ changes. The data also suggest that channel activation is not a simple step in the signal cascade initiated by PPI hydrolysis.(Supported by the C.N.R. Target Project on Biotechnology and Biostrumentation).

Protein Phosphorylation II

D 400 PKC AND MAP-2K ACTIVATION, Brian Akerley*, Carine Page, Lance Hultin, and Andre Nel. UCLA depts. of Microbiology and Immunology* and Medicine.
Ligation of the CD3 component of the TCR induces multiple signal transduction events which modify the activation state of the T-cell. We have compared two cell lines which express CD3 but vary in thier responses to ligation of this receptor. Jurkat cells respond to anti-CD3 with Ca++ mobilization, PKC activation, $p56_{lck}$ modification, and activation of a newly characterized 43kDa lymphoid microtubule associated protein-2 kinase (MAP2K). HPB-ALL cells were non-responsive in these assays but CD3 maintained induction of tyrosyl phosphorylation of at least one substrate. Moreover, either CD4 receptor in association with CD3 or direct chemical activation could restore some of these responses in HPB-ALL. Since it is also known that the CD3 receptor in HPB-ALL fails to induce IP_3 release we suggest that defective signal transduction by a G-binding protein could be responsible for the failure to induce PI turnover. This conclusion is further supported by the fact that artificial induction of PI turnover by exogenous phospholipase-C could activate these kinases in HPB-ALL. Although CD4 has been shown to upregulate induction of MAPK by the CD3 receptor in Jurkat cells, this receptor aggregate failed to induce MAP-2K activity in HPB-ALL cells. We did, however, observe increased phosphorylation of $p56_{lck}$ which coincided with increased association of p56lck with the CD4 receptor as determined by immune complex kinase assays. In contrast, PMA reduces the amount of $p56_{lck}$ activity which co-precipitates with the CD4 recptor and, interestingly, only under these circumstances does tyrosyl phosphorylation of p43 occurr in HPB-ALL. We therefore also suggest that PKC induced modification and intracellular redistribution of $p56_{lck}$ allows tyrosyl phosphorylation of MAP2K.

D 401 **THE REGULATION OF PROTEIN KINASE C ACTIVITY BY THE CD45 PHOSPHOTYROSINE PHOSPHATASE IN HUMAN T-CELLS. Denis Alexander and Emer Shivnan, Department of Immunology, Institute of Animal Physiology & Genetics Research, Babraham, Cambridge, CB2 4AT, .U.K.**

The role of the leucocyte common antigen (CD45) in regulating signal transduction events via the CD3 antigen has been investigated in human T-lymphocytes and in Jurkat T-leukaemia cells. The CD3 antigen consists of three (γ,δ,ε) out of the seven polypeptides which comprise the antigen receptor complex, and is thought to play a role in mediating signals arising from antigen binding. Cross-linking of CD45 with the CD3 antigen using biotinylated antibodies caused a marked inhibition in the TCR-mediated elevation in intracellular calcium. Utilising a novel protein kinase C (PKC) assay in which peptide substrates are introduced into streptolysin-O permeabilised cells (Alexander,D.R. et al.,Biochem.J.268,303-308,1990), the inhibition of CD3-mediated PKC activation by CD45-CD3 co-aggregation was also demonstrated. These findings were confirmed in intact [^{32}P]-labelled cells by demonstrating a reduction in the phosphorylation levels of 19kDa and 80kDa PKC protein substrates. Possible mechanisms of CD45 action are discussed.

D 402 **SOURCES OF INOSITOL TETRAKISPHOSPHATES IN WRK-1 CELLS.** Christopher J. Barker, Nai Sum Wong, Sarah H. Maccallum, Christopher J. Kirk & Robert H. Michell.

School of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham, B15 2TT. U.K.

It has now been shown that a number of species of inositol tetrakisphosphate (InsP_4) exist in mammalian cells. Ins(1,3,4,5)P_4 has a role as a co-messenger with Ins(1,4,5)P_3 in the regulation of intracellular calcium. Ins(1,3,4,6)P_4 is also formed on the stimulation by various agonists and would appear, in cell homogenates, to be synthesised by the phosphorylation of Ins(1,3,4)P_3: this however has been disputed *in vivo* (Stephens and Downes, 1990, Stephens *et al.*, 1990). A third compound, Ins(3,4,5,6)P_4, is the dominant InsP_4 in resting WRK-1 cells (Barker *et al.*, 1988) and it too accumulates in response to agonists known to activate phosphoinositidase C, though its origin is also unclear. Recent data suggest it may be elevated in cells which are over-expressing the src-oncogene (Johnson *et al.*, 1989). Experiments have been carried out to try and delineate the pathway of production of this InsP_4, and to relate it to the better understood inositol polyphosphates that are metabolic products of the key second messenger: Ins(1,4,5)P_3.

Barker. C.J., Morris, A.J., Kirk, C.J. & Michell, R.H. (1988) Biochem. Soc. Trans. 16, 984-985.

Johnson, R.M., Wasilenko, W.J., Mattingly, R.R., Weber, M.J. & Garrison, J.C. (1989) Science 246, 121-124.

Stephens, L.R. & Downes, C.P. (1990) Biochem. J. 265, 435-452.

Stephens, L.R. Berrie, C.P. & Irvine, R.F. (1990) Biochem. J. 269, 65-72.

D 403 INFLUENCE OF THE ANTIGENRECEPTORS ON THE DIFFERENTIATION OF B-LYMPHOCYTES

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Primary, murine B-lymphocytes (B-cells) can be stimulated by bacterial lipopolysaccharide (LPS) or Interleukin 5 (IL 5) to proliferate. Part of the cells eventually differentiate and secrete immunoglobulins (Ig). The differentiation process is characterized by a large increase in the amount of RNAs coding for the secreted form of IgM (μ_S, J-chain and κ RNA). When the membrane-bound IgM is crosslinked by high doses of $F(ab')_2$ fragments of antigenreceptor-antibodies (αμ) differentiation to Ig secretion does not occur, even if LPS or IL 5 is present in the culture. The proliferative response of the cells stays almost unaffected. At the RNA level, a specific loss of μ_S and J-chain RNA and a reduction of κ RNA can be observed. The inhibition of differentiation by αμ despite the presence of a potential differentiation signal can be mimicked by analoga of the hydrolysis-products of phosphatidyl-4,5-bisphosphate i.e. by phorboldibutyrate and ionomycin. Since long-lasting treatment of B-cells with these substances leads to a depletion of protein kinase C (PKC) after a strong initial activation (Mond et al.) and since Ig secretion and incorporation of ^{3}H-thymidine were determined 3 days after the beginning of stimulation the data suggest one of two possibilities: 1) the PKC might be involved in the differentiation process to Ig secretion and its exhaustion arrests this process, but not proliferation or 2) overstimulation of the PKC directly delivers a negative signal for differentiation.

Mond, J.J. et al. (1987) Proc. Natl. Acad. Sci. USA 84: 8588

D 404 OLIGOMERIZATION OF CD4 MOLECULE LEADS TO T CELL ANERGY,

Jacques Bernier, Sylvain Meloche, Didier Mary, Timothy Gregory and Rafick-P. Sékaly, Laboratory of Immunology, Institut de Recherches Cliniques de Montréal, Montréal, (Québec), Canada. H2W 1R7.

We have investigated the signalling function of the CD4 molecule. Monomerization, dimerization or oligomerization of CD4 was achieved in a population of purified human CD4$^+$ T cells. For this purpose, a chimeric molecule of HIV-1-gp120 containing 25 a.a. of glycoprotein D (gD) of herpes simplex virus at the NH_2-terminus was used. Dimerization was obtained with a specific mouse antibody against gD (5B6 mAb) and a goat anti-mouse antibody (GAM) was used to oligomerize the CD4-gp120-5B6 mAb complex. Cross-linking of human CD4 with gp120 and 5B6 mAb induced a slight increase of intracellular Ca^{++} influx, while oligomerization with GAM resulted in a marked increased in Ca^{++} influx. Ca^{++} mobilization was followed by an enhancement of IL-2 receptor, transferrin receptor and HLA-DR expression. This phenomenon is specific since it was dose-dependent and was inhibited by soluble recombinant CD4. Dimerization and oligomerization of CD4 lead to phosphorylation and activation of the CD4 associated tyrosine kinase $p56^{lck}$. Interestingly. the expression of IL-2 receptor did not lead to DNA synthesis. Moreover, addition of either exogenous IL-1 or IL-2 or a mitogenic dose of PMA did not drive the cells into S phase, suggesting that these T cells have been rendered anergic.
Results of the experiment demonstrated the negative signal transduced by CD4 molecule involves $p56^{lck}$. Furthermore these experiments provide a model system for the T cell anergy observed in AIDS patients.

D 405 INCREASED EXPRESSION OF PKCα AND DECREASED EXPRESSION OF PKCε IN CELLS TRANSFORMED BY RAS, FOS OR SRC ONCOGENES,

Christoph Bomer, Sarah Nichols Guadagno, Wendy Hsiao and I. Bernard Weinstein. Institute of Cancer Research, Columbia University, New York, NY 10032 and Department of Molecular Biology & Biochemistry, Irvine, CA 92717.

Derivatives of R6 embryo fibroblasts and K22 liver epithelial cells stably transformed by an activated c-H-ras oncogene display a several fold increase in expression of PKCα and a concomitant decrease in PKCε, both at the protein and mRNA levels. We now report similar changes in the expression of these two endogeneous PKC isoforms when the transformed phenotype is induced by Zn^{2+} in R6-cells carrying the activated ras oncogene under the control of a *metallothionein promoter. These experiments provide firm evidence that the increased expression of PKCα and the* decreased expression of PKCε are directly linked to the expression of the oncogenic Ras oncoprotein and are not due to secondary effects of the stably transformed cells due to clonal selection and further gene mutations. We extended our work to R6 cells expressing a high level of an exogeneously introduced PKCβI concomitant with various other oncogenes. Whereas in v-fos and v-src transformed cells PKCα was elevated and PKCε down-regulated this was not seen in cells transformed with v-myc, v-mos and activated neu. These results indicate that Src, Ras and Fos oncoproteins presumably via operating on a similar signal transduction pathway are capable of modulating PKCα and PKCε expression levels. It is possible that these changes contribute to the malignant cellular phenotype excerted by these oncogenes. Nuclear run-ons and actinomycin experiments will be presented to address the issue whether the changes in PKCα and PKCε expression in ras-, fos- and src-transformed cells occur on the transcriptional or post-transcriptional level.

D 406 Regulation of Plasma Membrane Phosphatidylinositol Kinase Activity,
Wendy F. Boss and Wolfgang Gross. Department of Botany, North Carolina State University, Raleigh, NC 27695
Higher plants contain phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-phosphate (PIP_2); however, the low concentration of PIP_2 (0.1 to 0.5 % of the total inositol phospholipid) and the high concentration of diacylglycerol (DAG) in the plasma membrane suggest that the primary role of PIP_2 may not be to serve as a source of the second messenger inositol trisphosphate and that DAG may not be a second messenger in higher plants. Alternatively PIP and PIP_2 can directly affect membrane structure and function. We have observed a rapid increase in PI and PIP kinase activity and a concomitant increase in the plasma membrane ATPase and phospholipase A_2 activity in response to treating carrot culture cells with cell wall degrading enzymes (e.g. hemicellulase, Chen and Boss, Plant Physiol., in press). The fact that the plasma membrane phospholipase A_2 activity is most sensitive to the wall degrading enzymes suggests that the stimulus response pathway in this system involves initial activation of the phospholipase A_2 followed by activation of the PI kinase. Experiments are in progress to subtantiate this pathway in vitro.

D 407 THE B CELL ANTIGEN RECEPTOR COMPLEX: LIGAND-INDUCED TYROSINE PHOSPHORYLATION OF THE α, β, AND γ COMPONENTS,
Kerry S. Campbell and John C. Cambier, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206

The major B cell antigen receptors consist of membrane-spanning forms of immunoglobulins M and D (mIgM and mIgD). Ligation of either mIgM or mIgD transduces an essential growth signal for B cell activation, and both receptors couple to phosphoinositide-specific phospholipase C and tyrosine kinase signalling pathways. However, mIgM and mIgD have minimal cytoplasmic structure (KVK in sequence on each of two membrane spanning heavy chains) and direct coupling to signalling structures seems unlikely. Recently, antigen receptor associated structures have been identified which may play a role in physical coupling to signal transduction structures, analogous to CD3 components in association with the T cell antigen receptor. The B cell antigen receptor complex contains α, β, and γ subunits which are protein products of the previously cloned genes, *mb-1* and B29. α, β, and γ are transmembrane glycoproteins which are disulfide-linked as heterodimers and non-covalently associated with mIgM and mIgD. Ligation of mIgM or mIgD with antibodies induces the tyrosine phosphorylation of α, β, and γ. Interestingly, two homology sequences are shared between MB-1 and B29 around intracellular tyrosine residues (YEGLN and YED). These homology sequences may act as consensus motifs for recognition by a tyrosine kinase or a tyrosine phosphatase. (Supported by NIH grant AI21768 and National Research Service Award AI08202).

D 408 A MANGANESE-DEPENDENT SERINE KINASE COPURIFIES WITH PHOSPHOINOSITIDE 3-KINASE.
Christopher L. Carpenter, Brian C. Duckworth, Kurt R. Auger and Lewis C. Cantley. Department of Physiology, Tufts University Medical School, Boston, MA 02111.
Phosphoinositide 3-kinase (PI 3-kinase) is involved in growth factor and oncogene signal transduction. The enzyme is a heterodimer of an 85 kDa and one of two related 110 kDa proteins. In vivo the 85 kDa subunit of PI 3-kinase is phosphorylated on tyrosine, serine and threonine. In looking for serine/threonine kinases that phosphorylate PI 3-kinase we discovered that a serine kinase copurifies with PI 3-kinase. Native isoelectric focusing gels show that the two enzymatic activities are present in the same complex. The serine kinase activity is manganese dependent. It phosphorylates both subunits of PI 3-kinase and will phosphorylate casein and histone. There is no major effect of serine phosphorylation of purified PI 3-kinase on its enzymatic activity. Since only the two subunits of the PI 3-kinase are visible on silver-stained SDS-PAGE the serine kinase is either a minor contaminant or is one of the subunits of PI 3-kinase.

D 409 **CYTOKINE PRODUCTION BY T CELLS: CD4 DEPENDENT AND INDEPENDENT CONTROL MECHANISMS** Narendra Chirmule, Naoki Oyaizu, Yoko Ohnishi and Savita Pahwa. Department of Pediatrics, North Shore University Hospital, Manhasset, NY 11030.
T lymphocytes secrete various cytokines upon antigenic stimulation. We have investigated the secretion of IL-2, IL-4, IL-6, TNF-α, and IFN-γ by antigen-specific CD4 positive T cell clones. The various cytokines secreted by the clones upon stimulation with specific antigen in presence of antigen presenting cells, were detected by ELISA/RIA, northern blot analysis and bioassays. To determine the influence of the CD4 molecule in controlling cytokine secretion, T cell clones were pretreated with anti-CD4 monoclonal antibodies (Leu 3a or OKT4a) or purified envelope glycoproteins of HIV-1, gp120. Treatment of the T cell clones with anti-CD4/gp120 inhibited the antigen-induced secretion of IL-2, IL-4 and IFN-γ in a dose dependent manner. Maximal inhibition was observed at 1 ug/ml of anti-CD4/gp120. This concentration was not toxic to the T cell clones as assessed by trypan blue exclusion. Induction of IL-6 and TNF-α were not inhibited by anti-CD4/gp120 pretreatment. Addition of cycloheximide to antigen stimulated cell cultures showed that the inhibition of cytokine secretion by anti-CD4/gp120 treatment was at the transcriptional level and not due to decreased stability of mRNA. Anti-CD4/gp120 pretreatment did not inhibit cytokine secretion by T cell clones upon stimulation via alternate pathways CD2, CD28 plus PMA or PMA plus ionomycin. These results demonstrate that specific antigen induced IL-2, IL-4 and IFN-γ secretion by CD4 positive T cells is dependent upon the presence of a functional CD4 receptor, but IL-6 and TNF-α secretion occurs by CD4 independent pathway(s) of T cell activation.

D 410 **THE NON-PHORBOL TUMOR PROMOTER OKADAIC ACID INCREASES EGF RECEPTOR LEVELS AND SENSITIZES RAT LIVER EPITHELIAL CELLS TO EGF-INDUCED DNA-SYNTHESIS. Nicholas M. Dean, Frank Y. Mukaida and Alton L. Boynton. Cancer Research Center of Hawaii, 1236 Lauhala St., Honolulu HI 96813.**
Okadaic acid (OA) is the first reported example of a compound exhibiting potent selective inhibitory activity towards protein serine/ threonine phosphatases and has also been shown to be a tumor promoter *in vivo* in mouse skin. We have therefore investigated the effect of OA on cell proliferation *in vitro* using the rat liver T51B epithelial cell line, which becomes density arrested and subsequently proliferates in response to EGF. Although itself not mitogenic, OA at low (10 nM) concentrations will potentiate the mitogenic effect of EGF. This is accompanied by an increase in EGF receptor number, EGF receptor protein and EGF dependent receptor autophosphorylation. This is the first example of receptor regulation by a phosphatase inhibitor, and represents a mechanism by which protein phosphatase inhibition can lead to the formation of tumors *in vivo*.

D 411 DEPENDENCE ON CALCIUM AND PROTEIN KINASE C FOR NATURAL KILLER (NK) FUNCTION BUT NOT NATURAL CYTOTOXIC (NC) FUNCTION IN A CLONED IL2-DEPENDENT CELL LINE. Julie Y. Djeu and Allen L. Richards. University of South Florida Coll. of Med., Dept. of Medical Microbiol. Tampa, FL 33612
Using a cloned murine cell line, NKB61A2 that concomitantly exhibits both NK and NC functions, we investigated the biochemical mechanisms involved in natural cell mediated cytotoxicity against NK-sensitive YAC-1 tumor cells and NC-sensitive WEHI tumor cells. To determine the role of calcium in NK and NC activities in the NKB61A2 cells, we evaluated the effect of 1) extracellular Ca++ depletion by the divalent cation chelator, EGTA, 2) blocking of intracellular Ca++ mobilization by 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8). We found that EGTA, verapamil and TMB-8 were all capable of inhibiting NK activity but they had little effect on NC activity of the cell line. Using 1-(5-isoquinolinylsulfonyl)-2 methylpiperazine (H-7) and N-(6-aminohexyl_-5-chloro-1-naphthalenesulfonamide (W-7) which are inhibitors of Protein Kinase C (PKC) and calmodulin respectiviely, we determined that PKC and calmodulin are vital in NK function but play no role in NC function. These data indicate that whereas NK activity is Ca++ and PKC dependent, NC is not, and may explain the disparate reports seen in the literature on calcium-dependent and independent lysis of tumor cells.

D 412 PROTEIN SYNTHESIS INHIBITORS DIFFERENTIALLY ELICIT MITOGEN-REGULATED INTRACELLULAR SIGNALS: AN HYPOTHESIS OF SUPERINDUCTION, Dylan R. Edwards[1] and Louis C. Mahadevan[2], [1]Department of Pharmacology, University of Calgary, Calgary, Alberta, Canada T2N 4N1, [2]Department of Biochemistry, Oxford University, Oxford, UK OX1 3QU
When quiescent cells are stimulated with growth factors such as epidermal growth factor (EGF) or with tumour promoters such as tetradecanoyl phorbol acetate (TPA), the proto-oncogenes c-fos and c-jun are rapidly and transiently induced. Addition of the protein synthesis inhibitors, anisomycin or cycloheximide along with EGF or TPA results in superinduction of c-fos and c-jun, a phenomenon that arises from a combination of increased transcription and increased mRNA stability. EGF and TPA elicit rapid phosphorylation of complexed and chromatin-associated forms of a 33kDa protein (pp33) and that of a 15kDa chromatin-associated protein (pp15). We show here that anisomycin and cycloheximide strongly stimulate pp33 and pp15 phosphorylation whereas puromycin produces an extremely weak response. All three agents produce virtually complete inhibition of protein synthesis. The capacity of these compounds to induce and, in combination with EGF, to superinduce c-fos and c-jun correlates not with their ability to inhibit protein synthesis, but with their ability to elicit and prolong pp33 and pp15 phosphorylation. We hypothesise, therefore, that the unique intrinsic ability of some protein synthesis inhibitors to elicit the same early nuclear signals as EGF and TPA contributes to their capacity to induce and superinduce c-fos and c-jun.
Supported by Alberta Heritage Foundation for Medical Research, Medical Research Council of Canada and Glaxo (U.K.)

D 413 SIGNAL TRANSDUCTION THROUGH THE HIGH AFFINITY IgE RECEPTOR IS MEDIATED BY THE LYN OR YES TYROSINE PROTEIN KINASES, Elisa Eiseman, Joseph B. Bolen, Laboratory of Tumor Virus Biology, National Cancer Institute, NIH, Bethesda, MD 20892.

The high affinity IgE receptor (FcεRI) expressed on the surface of basophils and mast cells plays a central role in immediate allergic responses. Analysis of FcεRI-mediated signal transduction in rat basophilic leukemia cells (RBL-2H3) and the mouse mast cell line PT18 suggests the involvement of protein tyrosine phosphorylation. Crosslinking of FcεRI in both of these cell lines with an anti-FcεRI monoclonal antibody, or by sensitizing with anti-2,4-dinitrophenol (DNP) mouse monoclonal IgE and then stimulating with antigen results in the tyrosine phosphorylation of several distinct proteins. Further analysis of this phenomenon suggests that the tyrosine kinase involved in FcεRI-mediated signaling in RBL-2H3 cells may be $p56^{lyn}$, the most abundant *src*-related tyrosine kinase expressed in these cells. The $p56^{lyn}$ kinase activity co-immunoprecipitates with FcεRI and becomes activated within one minute of receptor crosslinking. However, in PT18 cells the only detectable *src*-related tyrosine kinase is $p62^{c\text{-}yes}$, and it is this kinase activity that co-immunoprecipitates with FcεRI and is activated upon crosslinking of the receptor. These results suggest that at least two different *src*-related tyrosine kinases can associate with the same receptor and become activated after receptor-mediated signalling.

D 414 **cAMP IS NOT RESPONSIBLE FOR THE DIFFERENTIAL EFFECT OF CSA ON T CELL FUNCTIONS.** *A.ELJAAFARI, F.PAILLARD*, I.DORVAL. C.VAQUERO*, AND G.STERKERS. LABORATOIRE D'IMMUNOLOGIE, CJF 9015, HOPITAL ROBERT-DEBRE, 48 BD SERRURIER, 75019 PARIS, and * U152,INSERM HOPITAL COCHIN, 22 rue Mechain,75014 PARIS, FRANCE.*

Cyclosporine A (CSA), a cyclic undecapeptide of fungal origin is a powerful immunosuppressive drug. Similarly to cAMP, it inhibits helper function, i.e.: lymphokine production and proliferation, but affects only partially cytolytic activity on T lymphocytes. Thus, we assume that the differential effect observed on T cells after CSA treatment could be related to CSA-induced intracellular cAMP increament.

To test this hypothesis, we studied the effects of CSA on helper and cytolytic functions of a bifunctional T cell clone induced through CD3/TCR or CD2 activation pathways. Helper function was determined by studying autocrine mitosis and/or IL2, IL4, IFN gamma mRNA accumulation; Cytolytic activity was tested in a standard chromium release assay. In each condition of stimulation, CSA completely inhibited helper function but only weakly cytolytic activity. Same results were obtained with forskolin a potent activator of adenylcyclase. We then tested the effect of CSA on intracellular cAMP by using a RIA assay and did not observe any cAMP increament.

From these results, we can conclude that cAMP is not involved in the mechanism of action of CSA on T cells. Interestingly, by using a bifunctional T cell clone, we can infer that the differential effect of CSA or cAMP on T cell functions is due to different activation requirements, rather than to different sensitivity of T cell subsets to CSA or cAMP.

D 415 EFFECTS OF STAUROSPORINE AND DERIVATIVES ON PKC

Doriano Fabbro (1), Marlene Wolf (3), Ireos Filipuzzi (1), Christoph Borner (1), Roland Imber (1), Thomas Meyer (2), Alex Matter (2); 1:Laboratory for Molecular Tumorbiology, Department of Research, University Clinic, Basel and 2:CIBA-GEIGY, Pharmaceutical Division, Basel, Switzerland, 3:Theodor Kocher Institute, Bern, Switzerland.

Staurosporine (ST) and its selective derivative (CGP 41251) as well as its inactive derivative (CGP 42700) were analyzed with respect to inhbition of cellular PKC activity and TPA-induced phosphorylations in MDA-MB-231 cells. Both ST and CGP 41251 inhibited PKC activity of MDA-MB-231 cells with IC50 comparable to those found with purified PKC. In 32P-labeled MDA-MB-231 cells ST and CGP 41251 inhibited the TPA-dependent phosphorylation of the stress protein p27 with IC50s that were 10-fold higher than those required for the inhibition of intracellular PKC activity. In addition ST inhibited the basal TPA-independent phosphorylation of p16. The ST failed to inhibit the phosphorylation that converts the 74 kDαPKC to the 77 kDαPKC but caused a calcium-dependent translocation of PKC to MDA-MB-231 membranes. The ST-dependent translocation to membranes requires the holoenzyme of PKC as evidenced by calcium-dependent binding of purified PKC to erythrocyte inside-out vesicles. Thus, ST inhibited intracellular PKC activity and TPA-mediated phosphorylations but had additional effects on PKC and PKC-mediated responses.

D 416 HIGH-EFFICIENCY EXPRESSION OF PROTEIN KINASE C IN BACULOVIRUS-INFECTED INSECT CELLS, Bernd Fiebich, Dieter Marmé and Hubert Hug, University of Freiburg, Institute of Molecular Cell Biology, c/o Gödecke AG, Mooswaldallee 1-9, 7800 Freiburg, FRG

Protein kinase C (PKC) plays a central role in signal transduction. We have expressed rat PKC-γ (Knopf *et al.*, Cell 19: 491, 1986) in insect cells using the baculovirus vector pVL1393 (kindly provided by M. Summers). The yield of expressed PKC-γ is about 4% of total protein. The recombinant protein shows a prominent band at about 80 kDa on SDS-polyacrylamide gels, which can be identified by western blotting as PKC-γ by using monoclonal antibodies against PKC-γ. Upon incubation with [γ-^{32}P]ATP and in the presence of Ca^{2+}, phosphatidylserine and diacylglycerol this protein autophosphorylates. Its enzyme activity shows the characteristic properties of mammalien PKC.
Currently we are constructing recombinant baculoviruses containing cDNAs of human PKC-α (Finkenzeller *et al.*, NAR 18: 2183, 1990), PKC-ßI and PKC-ßII (Kubo *et al.*, FEBS Lett. 223: 138, 1987). The different biochemical properties of the expressed PKC isozymes will be compared.

D 417 EPIDERMAL GROWTH FACTOR STIMULATES PHOSPHATIDYLCHOLINE HYDROLYSIS, 1,2-DIACYLGLYCEROL FORMATION AND PROTEIN KINASE C ACTIVATION IN HUMAN DERMAL FIBROBLASTS. GJ Fisher, PA Henderson, JJ Voorhees, JJ Baldassare, Dept. of Derm., Univ. of Michigan, Ann Arbor, MI and Dept. Int. Med. St. Louis Univ., St. Louis, MO

We have investigated whether epidermal growth factor (EGF) induces formation of 1,2-diradylglycerol (DG) and activates protein kinase C (PK-C), in human dermal fibroblasts (HDF). HDF were treated with EGF (50ng/ml) for various times and DG mass measured by enzymatic assay. EGF elevated DG (2-fold) within 1 minute. Maximal accumulation (4-fold) ocurred at 5 minutes. Analysis of the linkage at the sn-1 position revealed that EGF induced accumulation of 1,2-diacylglycerol (DAG), exclusively. To determine the precursors and enzymatic pathways for EGF-induced DAG formation, cellular phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) were labelled with [^{3}H]choline, [^{3}H]ethanolamine, or [^{3}H]inositol, respectively, and the radioactive products formed in response to EGF measured. In cells labelled with [^{3}H]choline, PC hydrolysis products were increased 2-fold within 5 minutes of EGF addition. No hydrolysis of PE or PI was observed. Quantitation by enzymatic assay revealed equivalent elevations in the mass of choline phosphate and choline, the products of phospholipase C (PLC) and phospholipase D (PLD)-catalyzed PC hydrolysis, respectively. To assess the relative contributions of these two enzymes to DAG formation, HDF were treated with EGF in the presence of [^{14}C]ethanol (0.5%). Under these conditions, PLD catalyzes transphosphatidylation of PC to form phosphatidylethanol (P-ETOH), rather than phosphatidic acid (PA), which cannot be broken down by PA phosphohydrolase (PAP) to DAG. P-ETOH was increased nearly 5-fold, 5 minutes after addition of EGF. This did not, however, effect EGF-induced DAG accumulation at 5 minutes. Thus PLD/PAP does not appear to be a major pathway for EGF-induced DAG-formation. EGF-induced hydrolysis of PC may therefore occur predominently via PLC. EGF stimulated phosphorylation of the 80kDa PK-C substrate. Phosphorylation was detectable within 30 seconds and maximal at 1 minute after addition of EGF. Pretreatment of cells with TPA, which down regulated PK-C, resulted in loss of EGF-stimulated 80kDa phosphorylation. These data indicate that EGF increases DAG and activates PK-C in HDF.

D 418 V-SRC-INDUCED INCREASES IN DIACYLGLYCEROL DERIVE FROM PHOSPHATIDYLCHOLINE *via* A TYPE D PHOSPHOLIPASE ACTIVITY

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Activating the kinase activity of v-Src leads to activation of protein kinase C (PKC) (Spangler *et al.*, 1989. PNAS 86:7017). PKC is activated by diacylglycerol (DAG). If cells are labeled with [^{3}H]-arachidonic acid, we observe no increase in DAG production in response to v-Src. In contrast, if cells are labeled with [^{3}H]-glycerol, [3H]-myristate, or [^{3}H] palmitate, we see substantial increases in DAG production in response to v-Src. Myristate and palmitate are incorporated preferentially into phosphatidylcholine; arachidonic acid into phosphatidylinositol (Huang and Cabot, 1990. JBC 265:14858). This suggests that v-Src-induced DAG is derived from phosphatidylcholine. Consistent with this hypothesis, v-Src also induces increased levels of free choline with kinetics that correlate with DAG formation. Propanalol, which inhibits the conversion of phosphatidic acid (PA) to DAG, blocked v-Src-induced DAG production, and enhanced PA production. This suggests a type D phospholipase activity. Thus, v-Src-induced increases in DAG are likely the result of hydrolysis of phosphatidylcholine to PA and choline *via* a type D phospholipase, followed by the hydrolysis PA to DAG and phosphate.

D 419 CD28 STIMULATION INCREASES INTERLEUKIN 2 GENE TRANSCRIPTION BY A PATHWAY DISTINCT FROM THAT OF THE T CELL ANTIGEN RECEPTOR. James D. Fraser, Bryan A. Irving, Gerald R. Crabtree*, and Arthur Weiss, Howard Hughes Medical Institute, Departments of Medicine and Microbiology and Immunology, University of California, San Francisco CA 94143 and *Stanford Medical School and Howard Hughes Medical Institute, Stanford University, CA 94305.

CD28 is a 44 kD glycoprotein which is expressed as a homodimer on the majority of human T cells. Previous studies have demonstrated that binding of monoclonal antibody (mAb) 9.3 to CD28 can synergize with signals generated via the T cell antigen receptor (TCR) to induce T cell activation and lymphokine secretion. The signal transduction pathway of CD28 is currently unknown but appears to be distinct from that of the TCR. To investigate the mechanism of the CD28-generated augmentation of lymphokine production, we have used transient transfection assays of hybrid DNA constructs containing portions of the interleukin 2 (IL-2) gene enhancer region linked to the reporter genes chloramphenicol acetyltransferase and luciferase. While treatment with mAb 9.3 alone has no effect on IL-2 enhancer activity, the combination of PMA, ionomycin, and mAb 9.3 induces 4-6 fold greater reporter gene activity than does maximal stimulation with PMA and ionomycin alone. CD28 stimulation does not affect the enhancer activity of the previously characterized TCR-responsive NFAT 1 or NFIL2A elements. Analysis of fragments of the IL-2 enhancer indicate that the CD28-responsive element is located in the 3' region between -164 and -140 bp. Electrophoretic mobility shift assays of this region show novel DNA-protein complexes induced by mAb 9.3 treatment. Mutational analysis of this region indicate that the CD28-responsive element is distinct from the previously described NFIL2B element. These results indicate that CD28 stimulation increases IL-2 gene expression at least in part through an increase in the rate of transcription.

D 420 PHOSPHORYLATION OF THE MOS^{xe} PROTO-ONCOGENE DURING OOCYTE MATURATION. Robert S. Freeman and Daniel J. Donoghue. Department of Chemistry, University of California San Diego, La Jolla, CA 92093-0322.

Previous studies have demonstrated a role for the *mos* proto-oncogene in the meiotic maturation of oocytes. Microinjection of *Xenopus* oocytes with *in vitro* synthesized RNA encoding the *mos*^{xe} protein kinase induces oocyte maturation through an unknown mechanism that involves the activation of maturation promoting factor (MPF). Activation of MPF is known to involve changes in the phosphorylation state of both of the components of MPF: the *cdc*2 protein kinase and the cyclin B proteins. The *mos*^{xe} protein is itself phosphorylated during oocyte maturation. However, it is not known whether the phosphorylation of *mos*^{xe} regulates its protein kinase activity, nor whether it results from *mos*^{xe} autophosphorylation or from phosphorylation by another protein kinase. By studying the phosphorylation pattern of wild type *mos*^{xe} and a mutant inactive form of *mos*^{xe} (*mos*^{xeR90}), we have found that the eight most highly phosphorylated chymotryptic peptides are present in both the wild type and mutant *mos*^{xe} proteins. In addition, the wild type protein contains a few weakly phosphorylated peptides that must result from either *mos*^{xe} autophosphorylation or the activation of an unknown protein kinase during oocyte maturation. Phosphoamino acid analysis revealed that *mos*^{xe} is phosphorylated solely on serine residues. We are presently exploring whether the additional phosphorylation of wild type *mos*^{xe} is a consequence of autophosphorylation or of a maturation specific kinase. Also, we are attempting to map some of the most highly phosphorylated peptides in order to test these sites for a possible regulatory function.

D 421 PHOSPHATIDIC ACID - MEDIATED PATHWAY IS IMPORTANT IN PDGF - INDUCED MITOGENESIS. Kiyoko Fukami and Tadaomi Takenawa, Department of Pharmacology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, 173 Tokyo, Japan

PDGF-induced mitogenesis have been thought to be mediated by phosphatidyl-inositol turnover or/and receptor tyrosine kinase. Besides the formation of DG and IP_3, a great amount of phosphatidic acid (PA) was also accumulated during the stimulation of Balb/c 3T3 cells by PDGF. On the other hand, phospholipase D (PLD) but not PLC from bacteria induced the mitogenesis of the fibroblasts by the mechanism of PA formation. In addition, not only anti-PIP_2 antibody but also anti-PA antibody microinjected to the cells, not so strong as the former, inhibited the PDGF-induced mitogenesis.
As PA has been reported to inhibit the GAP activity, we found that PA stimulate the PI (3) kinase (type I). These data suggest that PA-mediated pathway may be important in the mitogenic signaling by PDGF.

D 422 ELEVATED LEVELS OF PKA ACTIVITY IN HUMAN BREAST CANCER, P Gazetas, C Borner, M Ueffing, J Pocsidio, P Logerfo, I.B.Weinstein, Comprehensive Cancer Center and Department of Surgery and Medicine, Columbia University, New York, NY.
The level of PKA activity in tumour cells has been found to be elevated or decreased as compared to normal tissue in different cell systems. PKA exists in two forms that can be chromatographically separated on DEAE and which differ in their regulatory subunit. Type II which appears in the .2M NaCl eluate, has been suggested to be involved in cellular differentiation; while Type I which is eluted with .1M NaCl, maybe related to cell prolifereation. Specimens of breast carcinoma and normal breast tissue from the same patient were collected from the operating room. Whole extracts as well as salt eluates were prepared. PKA activity in the whole extract was 1.3 to 6 fold higher in 13 of 15 patients. The activity of the Type I isozyme was 1.5 to 30 fold higher in all of 12 patients examined. However no difference in the level of activity of the Type II isozyme was found. Western and Northern blot analyses are being done. The PKA activity levels do not appear to correlate with several presently used prognostic indicators,ie. lymph node status, steroid receptor status, ploidy, or S-phase percentage. Further studies are in progress to evaluate the significance of the increased levels of PKA Type I isozyme in human breast carcinoma. (We thank Drs. A. Estabrook, S. Kister, and F. Gump for valuable assistance).

D 423 HERBIMYCIN A SPECIFICALLY INHIBITS TRANSMEMBRANE SIGNALLING VIA THE T CELL ANTIGEN RECEPTOR. Martha Graber*, Carl H. June+, Lawrence E. Samelson# and Arthur Weiss, Department of Medicine and Howard Hughes Medical Institute, University of California, San Francisco, San Francisco, CA., Immunobiology and Transplantation Department+, Naval Medical Research Institute, Bethesda, MD., and Cell Biology and Metabolism Branch#, National Institute of Child health and Human Development, National Institutes of Health, Bethesda MD.
Recent studies using the isoflavone derivative genistein, as well as studies of the tryrosine phosphatase CD45, and kinetic analyses of T cell transmembrane signalling, suggest that activation of phospholipase C (PLC) by the T cell antigen receptor (TCR) involves a regulatory tyrosine phosphorylation event. The human muscarinic receptor, type 1 (HM1) activates PLC via a G protein. In the T cell leukemic line Jurkat, transfected with HM1, PLC can be activated via both the TCR and the HM1 receptors. We have studied the effects of herbimycin A, a benzoquininoid ansamycin antibiotic which specifically down-regulates *src*-family tyrosine kinases, and of genistein, in this system. We show that herbimycin A, but not genistein, differentially inhibits PLC activation. Herbimycin A abolishes phosphoinositide hydrolysis and increase in intracellular free calcium induced by anti-TCR mAb, but does not inhibit these responses to the muscarinic agonist carbachol. Herbimycin A markedly diminishes the number and density of phosphotyrosine-containing bands in Western blots of whole cell lysates, in resting cells, and in cells stimulated with anti-TCR mAb, whereas treatment with genistein has no appreciable effect. Further evidence for the specificity of herbimycin A, and the lack of specificity of genistein, in this system is provided by the finding that both cell viability in overnight cultures, and uptake of ^{35}S-methionine into total cellular protein, were unaffected by herbimycin A, but were decreased by genistein in a dose-dependent manner, by 50% and 80% respectively. This study demonstrates that PLC may be activated by different mechanisms by two structurally disinct surface receptors in the same cell, and that these can be differentially inhibited by a reagent specific to the tyrosine kinase-mediated pathway.

D 424 SECRETION OF TRUNCATED AND CHIMAERIC IMMUNOGLOBULIN CHAINS IN BURKITT'S LYMPHOMA GENERATED BY CHROMOSOMAL TRANSLOCATIONS,

Peter Groitl, Günter Bernhardt and Martin Lipp, Institut für Biochemie, Ludwig-Maximilians-Universität, Karlstrasse 23, D-8000 Munich 2, FRG.

Variant chromosomal translocations in Burkitt's lymphoma (BL) have breakpoints located at variable distances downstream of the proto-oncogene *MYC* within a locus designated *PVT* but consistently upstream of immunoglobulin κ or λ light chain constant regions. It is unknown whether the translocations within *PVT* besides *MYC* deregulation contributes to tumourigenesis. We previously reported the cloning of 140 kb downstream of *MYC* encompassing several breakpoints of t(2;8) and t(8;22) translocations. Hybridization of non-repetitive probes from this region against RNA from several cell lines led to the identification of chimaeric RNAs transcribed across the breakpoints in BL lines and composed of sequences from chromosome 8 and from the constant segment of κ. The transcripts were cloned by screening cDNA libraries and by using the polymerase chain reaction on the first strand of cDNA derived from the cell lines with t(2;8) translocations. Some of the aberrant RNAs code for fusion proteins of different lengths containing the κ constant domain and various translated regions of chromosome 8. Although these fusion proteins are lacking a signal peptide they are secreted as proven by Western blotting with anti κ antibodies after *in vivo* expression. Furthermore we will present data about polyadenylated, non-chimaeric RNAs translated from the PVT locus. From these results we postulate that in BL the generation of chimaeric or truncated immunoglobuli, chains could possibly interfere with the signal transduction pathway in B cell differentiation.

D 425 A CALCIUM-UNRESPONSIVE PROTEIN KINASE C OF THE δTYPE

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A calcium-unresponsive protein kinase (PKC) was purified to homogeneity from porcine spleen. A characterization of this enzyme will be presented. The novel kinase is recognized by an antiserum raised in rabbits against a peptide of the COOH-terminus of δPKC. It is neither recognized by an α,ß,γ PKC-specific nor by an εPKC-specific antiserum. The δPKC-specific antiserum reacts also with respective kinases of other species. The apparent molecular weights of these kinases differ slightly (pig: 76 kDa, mouse and chicken: 82 kDa, fish: 84 kDa). The 82 kDa kinase (p82-kinase) is found in several murine tissues, predominantly in brain, lung, kidney, uterus and epidermis. Whereas α,ß,γ PKC is about equally distributed between the particulate and the soluble fraction of each tissue, the p82-kinase is almost exclusively located in the particulate fraction. Murine epidermis contains much more p82-kinase than α,ß,γ PKC. This is of special interest with respect to the phorbol ester-induced tumor promotion in mouse skin. Topical application of the phorbol ester TPA to mouse skin causes down-regulation of the epidermal p82-kinase, as determined by immunoblotting. 18 hours after TPA more than 80% of the enzyme is degraded and the recovery is very slow.

D 426 ANALYSIS OF TUMOUR NECROSIS FACTOR (TNFα and ß) AND IL1 SIGNAL TRANSDUCTION PATHWAYS IN HUMAN FIBROBLAST CELLS USING HIGH RESOLUTION 2-D GEL ELECTROPHORESIS. Graeme R. Guy and Carol Khong. Institute of Molecular Biology, 10 Kent Ridge Cres. National University of Singapore Singapore 0511. Primary human fibroblasts (MRHF), which have many types of growth factor and cytokine receptors on them, were pre-labelled with ^{32}P and stimulated for various times with differing doses of TNFα, TNFß and IL 1. The cell extracts were run on high resolution 2-D gel electrophoresis systems and the resulting autoradiographs were analysed by a Visage system. 200+ phosphorylated proteins could be seen on 12.5% gels. In most gels 20 to 30 proteins in agonist-stimulated samples were different from controls. Two of the proteins that show increased phosphorylation with TNF and IL1 were identified and have been previously cloned. The IL1 and TNF protein sets were compared to the phosphorylation patterns induced by agonists that transduce signals through partially known mechanisms; ionomycin, phorbol esters, cAMP activators, bradykinin and EGF. From the data it can be deduced that TNFs and IL1 share an identical signal transduction pathway (that was dissimilar to the other agonists) in the activation of a kinase(s) that may be unique to these agonists. The use of antibodies to TNF and IL1 showed the receptors to be operating through their respective receptors only. The genes induced by these agonists were also compared on 2-D gels after labelling cells with radioactive amino acids. In this system over 1600 proteins were visualised on each gel and again IL1 and TNF showed greater similarity in protein patterns compared with other agonists.

D 427 DIFFERENTIAL EFFECTS OF PROTEINKINASE INHIBITORS ON NGF-INDUCED NEURITE OUTGROWTH IN PC12 CELLS, Cornelia Hertel and Martine Nouiri, Pharma Research Department, F.Hoffmann-La Roche Ltd, 4002 Basel, Switzerland.

Staurosporine, K252a and H7 (and its analogues) represent two different classes of proteinkinase C (PKC) inhibitors, which are shown to inhibit ATP binding competitively. These compounds have been used previously to elucidate the involvement of PKC in NGF-induced neurite outgrowth, however, contradictory results have been observed.

Here we demonstrate that staurosporine and its analogue K252a inhibit NGF-induced neurite outgrowth at concentrations below K_i. At higher concentrations NGF-independent induction of neurite outgrowth was observed. In contrast H7 and its analogues potentiate NGF-induced neurite outgrowth with a relative potency as determined for PKC inhibition. These results suggest that the inhibitory effects of staurosporine may be due to inhibition of a highly sensitive kinase different from PKC, while H7 and its analogues may prevent NGF-induced downregulation by inhibiting translocation into the plasma membrane (Kondratyev et al., FEBS Lett. 264, 75-77 (1990)).

D 428 A GENE PRODUCT FROM YEAST ASSOCIATED WITH THE REPAIR OF DAMAGED DNA ENCODES A PROTEIN KINASE, Merl F. Hoekstra, Anthony DeMaggio, and Namrita Dhillon, Molecular Biology and Virology Lab, The Salk Institute, P.O. Box 85800, San Diego CA 92186-5800.

A wide variety of functions have been identified for repairing genotoxic damage. Such functions include the enzymatic and nucleolytic components involved in DNA repair while other functions are regulatory and presumably control the process. In smaller eukaryotes like *S. cerevisiae*, the *RAD* series of mutants are required for repairing UV and X-ray induced lesions and several *CDC* genes have also been implicated in DNA repair. We have used a genetic screen to identify genes in *S. cerevisiae* involved in repairing a site-specific double-strand break. One of these mutants, *hrr25-1*, is described in this report and shows a pleiomorphic phenotype. *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 the *HRR25* coding region result in additional phenotypes including cell cycle defects and aberrant cellular morphology. Surprisingly, the *HRR25* gene encodes a novel protein with two functional regions. The N-terminus of the protein encodes the kinase homology unit of approximately 250 residues. The C-terminus contains a repeated motif of 50 prolines and glutamines through the last 100 amino acids. The C-terminal domain appears to confer substrate recognition and/or intracellular localization. Potential activity of the Hrr25 kinase and its species distribution is being characterized.

D 429 ALTERATIONS OF THE LYMPHOCYTE-SPECIFIC PROTEIN TYROSINE KINASE, p56^{lck}, in response to IL-2. I.D. Horak[1,2], P.J. Lucas[3], E.M. Horak[1], B.Matoskova[1,2], R.E. Gress[3], J.B. Bolen[1]. Laboratory of Tumor Virus Biology[1], Medicine Branch[2], and Experimental Immunology Branch[3], National Cancer Institute, Bethesda, Maryland 20892.

T lymphocyte proliferation can be stimulated during T-cell activation as well as following the interaction of IL-2 with IL-2 receptor. The interaction of IL-2 with its receptors on the surface of T-cells results in rapid phosphorylation of several cellular proteins on tyrosine residues while other proteins are phosphorylated on serine and/or threonine residues. Whereas the IL-2 receptor complex on the surface of T-cells mediate these divergent phosphorylation reactions, no protein kinase has been found to be associated with either the p55 or p75 subunits of the IL-2 surface receptor. We have observed upon exposure of normal human T lymphocytes to IL-2 that specific acytivity of p56^{lck}, a membrane associated tyrosine protein kinase, is increased in a dose-and time-dependent manner. Addition of IL-2 was found to diminish the electrophoretic mobility of p56^{lck} on SDS polyacrylamide gels as the result of multiple phosphorylation alterations of the protein. These results indicate that IL-2 is capable of inducing changes in the activity and phosphorylation pattern of an abudant lymphocyte tyrosine protein kinase that has prioviously been shown to be involved in T-cell activation signal transduction pathways.

D 430 cAMP BLOCKS THE CELL CYCLE IN G1 AND G2/M IN IL-2 AND PMA INDUCED T-CELL LYMPHOCYTE PROLIFERATION, Richard L. Hurwitz, Dorothy Clark, Yung S. Yim, and Mary Y. Hurwitz, Dept. of Pediatrics and Cell Biology, Baylor College of Medicine, Houston TX 77030.
The effects of cyclic nucleotide analogs and IBMX, a phosphodiesterase inhibitor on the cell cycle of IL-2- or PMA-dependent T lymphocytes was explored. HT-2, a murine IL-2-dependent T cell line, was synchronized in G1 by incubating the cells without IL-2 or serum for 24 hours and verified by flow cytometry. The cultures were then supplemented with IL-2 (10 u/ml) or PMA (50 nM) and fetal calf serum (10%) in RPMI 1640 and incubated at 37°C in 5% CO_2. Aliquots of cells were taken at various time points and subjected to flow cytometry or pulsed with ^{3}H-thymidine. Viable cell numbers were determined using trypan blue exclusion. Cells began to enter S-phase by 6 hours following the addition of mitogen, peaked in S-phase by 18-24 hours and returned to G1 by 36-48 hours. The cell number doubled at approximately 36 hours. The addition of dibutyryl cAMP or 8-bromo cAMP (IC_{50}~25 µM) at the same time as the mitogen blocked the cells in G1. IBMX (IC_{50}~10 µM) had an identical effect. 8-bromo cGMP had no effect on the cell cycle or proliferation. The cells remained viable. When the cells were washed at 48 hours and media with mitogen but without cAMP analogs was added to the cells, the cells resumed entry into S-phase indicating that the inhibitory effects are reversible. When dibutyryl cAMP was added to the cultures 24 hours after the addition of mitogen (i.e. when the cells are in S-phase) and analyzed at 48 hours, the cells were found to be blocked at the G2/M boundary. This effect was not seen when IBMX was added to the culture. Again, the blockage at the G2/M boundary was reversible. Interpretations for the lack of an effect by IBMX at the G2/M boundary include: 1) a phosphodiesterase is not necessary for regulation of cAMP levels at this portion of the cell cycle, 2) IBMX cannot penetrate the cell compartment where the phosphodiesterase is present, or 3) IBMX does not inhibit the phosphodiesterase responsible for the regulation of cAMP levels at the G2/M boundary.

D 431 DIETHYLSTILBESTROL (DES) INDUCES PERSISTENT ACTIVATION OF PHOSPHATIDYLINOSITOL (PI) LIPID TURNOVER IN MOUSE UTERUS, D.M. Ignar-Trowbridge, A.R. Hughes, K.G. Nelson, J.W. Putney, K.S. Korach and J.A. McLachlan, Lab. of Reproductive and Developmental Toxicology and Lab. of Cellular and Molecular Pharmacology, Natl. Inst. Envir. Health Sci., Res. Tri. Park, NC 27709
There is considerable evidence that the mitogenic actions of estrogens in the uterus may be mediated to some extent by peptide growth factors. Many of the actions of growth factors have been associated with their effects on PI turnover and protein kinase C activation. Thus, the effect of in vivo estrogen treatment on PI lipid metabolism in the immature mouse uterus was investigated. We have previously shown that a prolonged elevation of PI lipid labeling with [^{3}H]myo-inositol occurs from as early as 1 hour until at least 18 hours after DES injection and this effect is associated with a substantial, time-dependent increase of the specific activity of both PIP and PIP_2. In vivo DES treatment is necessary for these effects since DES did not directly modulate PI lipid metabolism in vitro. We now report that the mass of IP_3 per uterus is elevated 12 hours after DES treatment. Furthermore, DES-induced elevation of PI lipid labeling is blocked by ICI 164,384, an estrogen receptor (ER) antagonist, which suggests that DES modulates PI lipid metabolism through ER mediated mechanisms. Thus, persistent activation of PI lipid metabolism may be an important component of estrogen-induced mitogenesis. Currently, estrogen effects on phosphorylation of cellular proteins crucial to signal transduction are being investigated.

D 432 PKC ISOFORMS IN AN IL-3-DEPENDENT MAST CELL LINE EXPRESSING ACTIVATED Ha-ras, Roland Imber, Ireos Filipuzzi, and Doriano Fabbro. Laboratory for Molecular Tumor Biology, Department of Research of Medical School, University of Basel, Switzerland.
Mutated Ha-ras caused tumorigenicity and a strong reduction of the IL-3 requirement of the mast cell line PB-3c. Cell lines expressing activated ras did neither reveal differences in the levels nor in the subcellular distribution of the PKC activity but displayed elevated amounts of immunoreactive β-PKC compared to the parental line. A reduction of the factor requirement similar to the one caused by ras could be achieved by the phorbol ester TPA. In addition, TPA and activated ras together led to a synergistic growth stimulus in the absence of IL-3 during a period of 72 hours. Upon TPA treatment a protracted down regulation of the immunodetectable.α-PKC were observed when oncogenic ras was expressed. These data suggest the involvement of specific PKC subtypes in the reduction of the IL-3 requirement caused by activated ras. Presence and subcellular distribution of various other PKC isoenzymes in these cell lines are described.

D 433 TRANSFORMATION-SENSITIVE CYTOSKELETAL ASSOCIATION OF α-PKC, Susan Jaken, Susannah Hyatt and Lan Liao, W. Alton Jones Cell Science Center, Lake Placid, NY 12946
Immunocytofluorescence studies have demonstrated that α-PKC is concentrated in focal contacts of normal but not SV40-transformed REF52 cells (Mol. Carcinogenesis, 3:45-53, 1990). To determine if there are cellular determinants of α-PKC subcellular localization, we developed and characterized an overlay assay to detect α-PKC binding proteins. The molecular interaction between α-PKC and these proteins depended on phospholipid. Phorbol esters decreased the calcium requirement for binding. Vinculin and talin, two focal contact proteins which are known to be PKC substrates, were also α-PKC binding proteins. The two major binding proteins from REF52 cytosols (p71 and p>200) were missing from extracts of SV40-REF52 cells. Thus, these two proteins are candidates for transformation-sensitive mediators of PKC-association with the cytoskeleton. Localization of α-PKC to the focal contacts of normal but not transformed cells suggests a functional role in regulating cell attachment and/or migration. In support of this, α-PKC rapidly accumulates on the leading lamellipodia of cells bordering a wound (scrape) site. We introduced anti-α-PKC neutralizing antibodies and pseudosubstrate peptide into living cells. Neither anti-catalytic nor anti-regulatory domain antibodies inhibited cell attachment, growth, or migration into a wound site. Pseudosubstrate peptide did not inhibit cell attachment, but did inhibit growth and migration. Down modulation also inhibited migration. These results indicate a role for PKC, although probably not α-PKC, in focal contact function.

D 434 IDENTIFICATION OF TARGET PROTEINS OF PROTEIN KINASE C IN MOUSE SKIN IN VIVO AND EPIDERMAL KERATINOCYTES IN CULTURE, Kohji Kasahara, Makoto Tsunenaga, Yohko Kohno, Tohgo Ikuta, Kazuhiro Chida, and Toshio Kuroki, Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo-108, Japan.
We are investigating signal transduction and gene expression in the stage of tumor promotion/progression of mouse skin carcinogenesis. We found that epidermal proteins with a molecular weight of 40K and 34K (p40 and p34, respectively) were phosphorylated mouse skin in vivo by protein kinase C (PKC), while BALB/MK-2 cells contained another three target proteins (i.e., p80, p70 and p28).
- p80 was a major phosphorylated acidic protein previously reported with Swiss 3T3 cells (MARCKS protein).
- p70 was identified as nuclear envelope lamin B from the data of co-migration, immunoblotting and peptide mapping.
- p40 was purified from mouse brain and identified as creatine phosphokinase B (CKB). Phosphorylation of CKB by PKC increased its activity both in intact cells and in cell-free system, owing to increase in its affinity for phosphocreatine, a substrate of the enzyme.
- p34 was purified from mouse brain but no homology exists with known proteins.
- p28 has not been identified.

D 435 INITIAL CHARACTERIZATION OF A NON-TRANSFORMED VARIANT OF RAT 6-PKC-3 CELLS, Robert S. Krauss, Sarah N. Guadagno and I. Bernard Weinstein, Institute of Cancer Research, Columbia University, New York, NY 10032.
Rat 6 cells that overproduce protein kinase C (PKC; R6-PKC-3 cells) form small colonies in 0.3% agar, large colonies in agar supplemented with the phorbol ester TPA and are hypersensitive to complete transformation by the T24 H-ras oncogene. Curiously, T24-transformed R6-PKC-3 cells are killed when exposed to TPA. We have introduced an inducible mouse metallothionein I (MT I) promoter-T24 H-ras construct into R6-PKC-3 cells and isolated a clone (A5) that exhibits a $ZnSO_4$-dependent fully transformed phenotype. Non-transformed revertants of A5 cells were isolated by selection for cells surviving a combination of induction of the H-ras oncogene to levels that are toxic to A5 cells plus treatment with TPA. One of these lines (R-1a) has a slow growth rate and does not grow in 0.3% agar or agar supplemented with TPA or $ZnSO_4$. However, R-1a cells express very high levels of PKC enzyme activity, show an exaggerated morphological response to TPA and still condition their medium with a PKC-induced growth factor. Additionally, these cells do not induce the MT I promoter-T24 H-ras construct or the endogenous MT I gene upon exposure to $ZnSO_4$. Defects in a specific PKC-mediated signal pathway in R-1a cells may lead to both lack of activity of the MT I promoter and loss of the transformed phenotype. Studies aimed at clarifying this point, as well as further characterization of R-1a cells, are underway.

D 436 PREDOMINANT EXPRESSION OF Ca^{2+}-INDEPENDENT PROTEIN KINASE C ISOFORMS, nPKCη AND nPKCδ IN MOUSE SKIN, Toshio Kuroki, Yu Hashimoto, Shin-ichi Osada, Osamu Tajima, Kiyoshi Nose, and Shigeo Ohno*, Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108, and *Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113, Japan.
Protein kinase C (PKC) molecules consist of a protein family which can be classified into two major groups, conventional PKC and novel PKC (nPKC). Conventional PKC isoforms (PKCα, βI, βII, γ) are characterized by the requirement of Ca^{2+} , phospholipids and diacylglycerol for activation and by the presence of three conserved domains, C1, C2 and C3, while nPKC isoforms (δ, ε, ζ, η) are independent of Ca^{2+} for activation and lacks the C2 domain. By the use of newly cloned mouse probes, we studied expression of PKC isoforms in mouse skin *in vivo*, a target tissue of phorbol ester tumor promoters, e.g. TPA. We found predominant expression of nPKCη, a new nPKC isoform isolated from mouse skin cDNA library showing skin- and lung-specific expression (see Osada et al. in this symposium). Higher expression of nPKCδ was also observed. However, PKCα, PKCβ(I+II), nPKCε and nPKCζ were expressed at very low level and detected only when poly(A)$^{+}$ RNAs were used. There was no signal for PKCγ. The present study indicates that nPKCη and nPKCδ are predominant isoforms in mouse skin and may have crucial roles in epidermal cell functions and also in tumor promotion.

D 437 ANTIGEN-INDUCED Fc RECEPTOR-DEPENDENT AND -INDEPENDENT B CELL DESENSITIZATION DOES NOT INVOLVE EITHER AN ELEVATION IN $[Ca^{++}]_i$ OR PROTEIN KINASE C ACTIVATION, Alan H. Lazarus, #Gordon B. Mills, Andrew R. Crow, and, Terry L. Delovitch, Banting & Best Department of Medical Research & Department of Immununology, University of Toronto, and #Oncology Research, Toronto General Hospital, Toronto, Ontario, M5G 2C4, CANADA
Interaction of Ag with surface Ig (sIg) on a B cell induces transmembrane signaling leading to an elevation in the concentration of intracellular free calcium $[Ca^{++}]_i$. This interaction also elicits a refractory state in which the B cell cannot respond to a second stimulus, a process referred to as desensitization. B cells can also be desensitized by crosslinking sIg to the $Fc_{\gamma}R$ via the formation of an Ag-Ab bridge. We previously found that transfected TNP-specific B cells undergo both calcium signaling and desensitization upon interaction with the thymus-dependent Ag TNP-OVA. In addition, crosslinking of sIg to FcR by TNP-OVA and anti-OVA Ab in these cells results in a desensitized state that is even greater than that obtained with Ag alone. Herein, we demonstrate that Ag-induced changes in $[Ca^{++}]_i$ are neither related nor sufficient for B cell desensitization. FcR-dependent desensitization also does not require a change in $[Ca^{++}]_i$. To investigate the role of protein kinase C (PKC) in B cell desensitization mediated by either Ag or sIg-FcR interaction, PKC was downregulated by long term exposure to TPA or inhibited by staurosporine. These PKC downregulated or inhibited cells underwent similar Ag- and FcR-dependent desensitization compared to control cells. These data indicate that Ag-induced desensitization of B cell signaling likely involves an event(s) that occurs either upstream or independent of Ag-induced elevations in $[Ca^{++}]_i$ and PKC activation.

D 438 DISSOCIATION OF PROTEIN KINASE C ACTIVATION AND *sn*-1,2-DIACYLGLYCEROL FORMATION: COMPARISON OF PHOSPHATIDYLINOSITOL- AND PHOSPHATIDYLCHOLINE-DERIVED DIGLYCERIDES IN α-THROMBIN-STIMULATED FIBROBLASTS, Karen L. Leach*, Valerie A. Ruff*, Timothy M. Wright†, Melissa S. Pessin‡ and Daniel M. Raben§, Dept. of Cell Biology*, The Upjohn Co., Kalamazoo, MI 49007; Dept. of Medicine†, Dept. of Physiology‡, Dept. of Neuroscience§, Johns Hopkins University School of Medicine, Baltimore, MD 21205
Diacylglycerols (DAGs) derived from phosphatidylcholine (PC) hydrolysis have been shown to activate protein kinase C (PKC) *in vitro*, but it is not known whether this event occurs in response to DAGs generated via agonist-induced PC hydrolysis in intact cells. In these studies we have addressed this question directly, using α-thrombin stimulation of IIC9 cells. PKC activation in intact cells was assessed in two ways, by measuring: 1) PKC membrane association as determined by kinase activity and Western blot analysis and 2) the phosphorylation of an endogenous PKC substrate, an 80 kD protein. Following treatment with 500 ng/ml α-thrombin, phosphoinositide (PI) hydrolysis is stimulated rapidly, leading to a peak in DAG levels at 15 sec. A second peak of DAG, which reaches a maximum at 5 min, is also generated in response to high concentrations of α-thrombin. Molecular species analysis has shown that the second peak of DAG results primarily, if not exclusively, from PC hydrolysis. In contrast, treatment with a low concentration of α-thrombin, 100 pg/ml, results in a monophasic generation of DAGs which peaks at 5 min. This peak of DAGs is generated primarily from PC hydrolysis. In cells treated with 500 ng/ml α-thrombin, PKC membrane association increased rapidly, and reached a maximum at 30 sec. By 15 min, the level of membrane PKC was the same as in the control cells. Stimulation of 80 kD phosphorylation by 500 ng/ml α-thrombin followed the same time course as PKC membrane association. These results demonstrate that PKC activation corresponded kinetically to the PI-generated DAG peak. In contrast, following treatment with 100 pg/ml α-thrombin, PKC membrane activity was not increased above the control value at any time during the 30 min time course. Similarly, this low concentration of α-thrombin did not stimulate 80 kD phosphorylation at any time point. Our results demonstrate, using two different criteria, that DAG produced from PI, but not PC hydrolysis, is associated with the activation of PKC in intact cells.

D 439 CROSS-LINKING OF TCR/CD3 OR CD2 STIMULATES THE APPEARANCE OF A SIMILAR PATTERN OF TYROSINE PHOSPHORYLATED PROTEINS,
Steven C. Ley, Adelina A. Davies and Michael J. Crumpton, Cell Surface Biochemistry Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, England.
Pharmacological and genetic experiments have demonstrated that tyrosine phosphorylation is important in signalling via the TCR/CD3 complex. However, it is not clear which tyrosine phosphorylated protein substrates are important in this signalling pathway. To identify candidate substrates, Jurkat T cells were stimulated with an anti-CD3 antibody and tyrosine phosphorylated proteins identified by immunoblotting with an anti-phosphotyrosine antibody. Twelve bands were identified, ranging in molecular weight from 21 KD to 180 KD. The major bands at 89, 70, 60 and 40 KD were detectable only 15 seconds after stimulation, reaching maximum intensity at 5 minutes and then declining to background levels by 60 minutes. Mitogenic combinations of antibodies to CD2 stimulated the appearance of a pattern of tyrosine phosphorylated which was indistinguishable from that stimulated by CD3 cross-linking. These data imply that CD2 may utilize the same tyrosine kinase signalling pathway as the TCR/CD3 complex.

D 440 PDGF AND EGF INDUCE BOTH THE RELEASE OF ARACHIDONIC ACID AND THE PHOSPHORYLATION OF cPLA$_2$,
Lih-Ling Lin, Alice Y. Lin and John L. Knopf, Department of Drug Discovery, Genetics Institute Inc., 87 CambridgePark Drive, Cambridge, MA 02142
The synthesis of the two potent inflammatory mediators, prostaglandins and leukotrienes, is regulated by the availability of free arachidonic acid. A cytosolic phospholipase A$_2$ (cPLA$_2$) catalyzes the cleavage of arachidonic acid from membrane phospholipids, and therefore plays a central role in the inflammation process. Two growth factors, PDGF and EGF, have been shown to cause the release of arachidonic acid and increased production of PGE$_2$, probably by activating a cPLA$_2$. Recently we have purified a 110 kDa cytosolic PLA$_2$ from U937 cells (1), and have subsequently raised cPLA$_2$ specific antisera. Here we demonstrate that treatment of RAT-2 fibroblasts with PDGF, EGF, or TPA increases the phosphorylation of cPLA$_2$ on serine residues by several fold. Moreover, the kinetics and degree of phosphorylation correlate with arachidonate release. These data suggest that phosphorylation of cPLA$_2$ by a serine kinase, not a tyrosine kinase, may activate cPLA$_2$ *in vivo*.
(1) J.D. Clark, N. Milona and J.L. Knopf. PNAS. 1990 in press.

D 441 Bombesin stimulation of *c-fos* expression and mitogenesis in Swiss 3T3 cells: The role of prostaglandin E$_2$-dependent cyclic AMP.
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Bombesin is a potent mitogen for Swiss 3T3 cells and can stimulate mitogenesis in the absence of any other growth factor. This effect is mediated by multiple synergistic signal transduction events which include an increase in both intracellular cAMP levels (mediated primarily by PGE$_2$ release) and *c-fos* mRNA expression. Indomethacin, an inhibitor of cyclooxygenase activity, abolished PGE$_2$ release and substantially depressed cAMP levels in Swiss 3T3 cells treated with bombesin. In contrast, the drug did not affect 80K phosphorylation or Ca^{2+} mobilisation by bombesin in parallel cultures, indicating that cAMP synthesis can occur through a phospholipase C-independent pathway. Indomethacin also caused a 30% decrease in *c-fos* induction and DNA synthesis in cells stimulated by bombesin. This inhibitory effect was reversed in the presence of forskolin, a direct activator of adenylate cyclase. We conclude that cAMP plays a regulatory role in *c-fos* induction and mitogenesis in Swiss 3T3 cells.

D 442 ACTIVATION OF PROTEIN KINASES IN WILD-TYPE AND PHORBOL ESTER-RESISTANT EL4 THYMOMA CELLS, Kathryn E. Meier, Karen A. Licciardi, Timothy A.J. Haystead, and Edwin G. Krebs, Dept. of Pharmacology, Univ. of Washington, Seattle, WA 98195.

In this study, we used the murine EL4 thymoma cell line to study the potential role of messenger-independent protein serine/threonine kinases that are activated downstream of protein kinase C [PKC] in cellular responses to phorbol ester (PMA). Addition of PMA to wild-type (WT) EL4 cells results in activation of a myelin basic protein (MBP) kinase and an RRLSSLRA peptide (S6P) kinase, as detected in cytosolic extracts prepared from these cells. These kinases are activated within 5 minutes after PMA addition and remain activated for at least 20 minutes. In a variant EL4 cell line in which PMA does not induce IL-2 transcription, PMA fails to activate either the S6P kinase or MBP kinase. The α isoform of PKC is expressed by both the WT and variant cells. PKC associates with the membrane fraction following exposure of either cell line to PMA. In intact cells, PMA treatment results in phosphorylation of some of the same protein substrates in both cell lines. Okadaic acid (OA) (10 μM), a phosphatase inhibitor, increased S6P and MBP kinase activities in both the WT and variant cells. In WT cells (but not in variant cells), 1 μM OA acts synergistically with PMA. The variant cells therefore appear to be defective in an intermediate step of a PKC-initiated phosphorylation cascade. These results suggest that activation of downstream protein kinases is required for some of the cellular responses that occur in response to phorbol esters.

D 443 OVEREXPRESSION OF SRF AND CREB IN BACULOVIRUS, Cindy K. Miranti and Michael Greenberg, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115

Stimulation of PC12 cells with NGF or membrane depolarizing agents immediately induces c-fos gene transcription. In these cells NGF stimulates a short proliferative response followed by differentiation into sympathetic neuron-like cells. Depolarization with elevated levels of KCl alters membrane potential by activating Ca++ channels, but does not induce proliferation or differentiation. While both agents induce c-fos transcription, they do so differently. NGF action is mediated by a transcriptional regulator, serum-reponse factor (SRF), which binds to the serum-response element located at -300 upstream of the c-fos gene and depolarizing agents mediate induction through the cyclic AMP element binding protein (CREB) which binds to the Ca++/cAMP-repsonse element located at -65. c-fos induction occurs in the absence of new protein synthesis and both SRF and CREB appear to bind constitutively to their respective regulatory elements. SRF and CREB are phosphoproteins, and CREB is specifically phosphorylated by CAM kinase II. Therefore, phosphorylation has been proposed as a likely mechanism of regulation. Additionally both of these elements can mediate the rapid down regulation of c-fos transcription, but the mechanism by which this occurs is not known. To investigate the role of SRF and CREB phosphorylation in the regulation of c-fos we isolated baculovirus recombinants overexpressing SRF and CREB. Recombinants were identified by western blots with specific antisera, and conditions determined for maximal expression. Proteins are currently being purified by DNA affinity chromatography. Investigations into the sites of phosphorylation and their effect on c-fos transcription in vitro will be presented.

D 444 PROTEIN KINASE C REGULATES BOTH PRODUCTION AND SECRETION OF INTERLEUKIN 2, Jaime F. Modiano, Rebecca Kolp, Roberta J. Lamb, and Peter C. Nowell, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, 19104.

Inhibiting protein kinase C (PKC) activity abrogated interleukin 2 (IL2) production by mitogen-stimulated human T lymphocytes. This effect was due partially to a 50% decrease in IL2 gene expression. However, when PKC inhibitors were added after IL2 gene transcription had already proceeded for 3 to 4 hours, the IL2 in the culture supernatants was still reduced by 30-80%, and intracellular IL2 was increased by up to 50%. The inhibition of PKC affected the expression of IL2 receptors by these cells differently: it had little effect on gene expression or on the membrane-bound form of the receptor, but it decreased soluble receptors in the supernatants by 50-80%. These data indicate that in addition to its previously defined role in gene expression, PKC can also regulate extracellular secretion of proteins critical for T cell proliferation.

D 445 PHOSPHATIDATE PHOSPHATASE FROM *SACCHAROMYCES CEREVISIAE*: ISOLATION OF 45-kDa AND 104-kDa FORMS OF THE ENZYME THAT ARE DIFFERENTIALLY REGULATED BY INOSITOL, Kelly R. Morlock, Jennifer J. McLaughlin, Yi-Ping Lin, and George M. Carman, Department of Food Science, Cook College, Rutgers University, New Brunswick, New Jersey 08903

PA phosphatase plays a role in animal cell signaling mechanisms as part of the phospholipase D-PA phosphatase pathway for the generation of DG from PC. The DG derived from PC is responsible for sustained activation of protein kinase C. Immunoblot analysis of cell extracts using antibodies specific for the 91-kDa form of membrane-associated phosphatidate phosphatase revealed the existence of a 45-kDa form of the enzyme. Immunoblot analysis also showed that the 91-kDa form of the enzyme was a proteolytic product of a 104-kDa enzyme. The enzymological properties of the 45-kDa and 104-kDa forms of the enzymes were similar. However, the phosphatidate phosphatase 45-kDa and 104-kDa proteins differed with respect to their isoelectric points, and peptide fragments resulting from V8 proteolysis and cyanogen bromide cleavage. The expression of the phosphatidate phosphatase 45-kDa and 104-kDa enzymes were differentially regulated in cells supplemented with inositol and both forms were induced when cells entered the stationary phase of growth.

D 446 THE 68kDa CALCIUM-BINDING PROTEIN, ANNEXIN VI, IS PHOSPHORYLATED IN A CELL CYCLE DEPENDENT MANNER, Stephen E. Moss and Michael J. Crumpton*, Department of Physiology, University College London, Gower Street, London WC1E 6BT, U.K., and *Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, U.K.
The annexin family of Ca^{2+}/phospholipid-binding proteins includes lipocortin (annexin I) and calpactin (annexin II), the major cellular substrates for phosphorylation by the EGF receptor and $pp60^{v-src}$ tyrosine kinase activities respectively. We report that annexin VI (p68, 67kDa-calelectrin) is also phosphorylated in intact cells, but that in contrast to the phosphorylation of annexins I and II, annexin VI phosphorylation is cell cycle dependent. In both Swiss 3T3 fibroblasts and human T-lymphoblasts, annexin VI is phosphorylated at low levels during mid-G1, and strongly during S/G2/M. During these stages of the cell cycle, annexin VI phosphorylation occurs on serine and threonine. The results provide a link between the annexins and the cell cycle, and more specifically suggest that annexin VI may be functionally regulated during the cell cycle.

D 447 CONTROL OF PROTEIN PHOSPHATASE 2A BY SIMIAN VIRUS 40 SMALL T ANTIGEN, Marc Mumby, Sung-Il Yang, Ronald Lickteig, Kathleen Rundell, Karl Heinz Scheidtmann, and Gernot Walter, Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX 75235-9041

Polyoma medium T (the transforming protein of this virus), and both polyoma and SV40 small-t antigens, form complexes with the serine/threonine-specific protein phosphatase 2A (PP2A). Experiments with medium T mutants suggest that complex formation with PP2A plays a role in transformation. Although the small-t antigens are not transforming by themselves, they support or enhance transformation under certain conditions.

Soluble, monomeric SV40 small-t antigen (small-t) was purified from bacteria and assayed for its ability to form complexes with protein phosphatase 2A. Three different multimeric forms of PP2A were used, composed of different combinations of regulatory (A and B) and a common catalytic subunit (C). Only the AC complex associated with small-t, as demonstrated by non-denaturing polyacrylamide gel electrophoresis. The purified A subunit also associated with small-t indicating that the primary interaction between small-t and PP2A is through the A subunit. The effect of small-t on phosphatase activity was determined using several exogenous substrates as well as SV40 large T antigen (large T) and the p53 anti-oncogene protein phosphorylated *in vivo*. Small-t specifically inhibited the activity of the AC form of PP2A with most substrates. A maximal inhibition of 50% was observed that had an IC_{50} of 14 nM small-t. The dephosphorylation of individual phosphorylation sites of large T and p53 were determined by two-dimensional peptide mapping. Individual sites within large T and p53 were dephosphorylated at different rates by all three forms of phosphatase 2A. The phosphates at Ser-120 and Ser-123 of large T, which affect the origin binding and SV40 DNA replicating activities, were removed most rapidly. PP2A specifically dephosphorylated 3 of the 6 major phosphorylation sites of p53. Dephosphorylation of most of the sites in large T and p53 by the AC complex was inhibited by small-t. The inhibitory effect of small-t on protein phosphatase 2A could explain the role of this protein in transformation.

D 448 REGULATION OF MARCKS PROTEIN AND mRNA LEVELS IN CELL LINES WHICH OVER-EXPRESS PKCβ_I, **Sarah Nichols Guadagno, Christoph Borner and I.Bernard Weinstein, Institute of Cancer Research, Columbia University, New York, NY 10032.**

Recently our laboratory has used retroviral infection to generate a Rat-6 cell line which overexpresses the βI isoform of PKC (R6-PKC3). This cell line exhibits a number of disorders in growth control, and hypersensitivity to complete transformation by an activated c-H-*ras* oncogene. To further examine alterations in signal transduction mediated by PKCβ_I, we have studied the effects of PKC overexpression on a well characterized PKC substrate, MARCKS (formerly 80/87 kD protein). We have studied a number of properties of this protein including its phosphorylation state, total protein levels, subcellular distribution of the protein, and mRNA levels. When compared to a vector control line (R6-C1), unstimulated R6-PKC3 cells show a small but reproducible increase in the level of phosphorylation of the MARCKS protein, as well as an increase in the total amount of MARCKS protein and mRNA. Following treatment with 100nM or 300nM TPA, both the C1 and PKC3 cells respond by elevating (2-3 fold) the total amount of immunodetectable MARCKS protein. The cytosolic levels of the protein are also increased dramatically following TPA treatment. For the R6-C1 cells, the elevation in total and cytosolic MARCKS protein can be detected at 30' and 6 hr following TPA treatment; however, by 24 hr both the total and cytosolic levels of the protein have decreased slighlty below the control level. On the other hand, PKC3 cells show elevated levels of total and cytosolic MARCKS protein for up to 24 hr following TPA treatment. These pronounced changes in protein levels were also seen in the PKC3 cells but not the C1 cells at very low doses of TPA (10nM). The increase in MARCKS detected at the protein level following TPA treatment is not reflected at the mRNA level. At all three doses of TPA tested (10, 100 & 300nM) the PKC3 cells showed a dramatic reduction in MARCKS mRNA levels following 1hr of TPA treatment and persisting for up to 24 hr. The C1 cells displayed no change in the MARCKS mRNA level at any dose of TPA tested. We are currently investigating the mechanisms underlying the changes we see in the MARCKS protein and mRNA in the PKC3 cells.

D 449 STABLE EXPRESSION OF REGULATORY DOMAIN DELETION MUTANTS OF PKC β_1 IN RAT FIBROBLASTS Kevin O'Driscoll, Scott M. Kahn, Christoph Borner, Wei Jiang and I. Bernard Weinstein. Institute of Cancer Research, College of Physicians and Surgeons, Columbia University, 701 West 168th Street, New York, NY. 10032

Previous studies from our laboratory have shown that the stable overproduction of protein kinase C (PKC) β_1 in rodent fibroblast cell lines is a useful model system to study the role of PKC in multistage carcinogenesis (Housey *et al.* Cell, 1988; Krauss *et al.* Oncogene 1989; Hsiao *et al.* Mol. Cell. Biol. 1989). To delineate protein domains within the primary sequence of PKC β_1 that may contribute to the action of this enzyme in altering cellular growth properties, deletion mutants were expressed stably in Rat6 fibroblasts. Precisely defined deletion mutants within the regulatory domain of the PKC β_1 cDNA coding sequence were generated using a polymerase chain reaction-based technique termed thermal cycled fusion (Kahn *et al.* Technique 1990). Mutant cDNA's lacking: *i.*) one of the two zinc finger-like, cysteine-rich protein regions; *ii.*) the C1 region including the pseudosubstrate peptide sequence, and both cysteine-rich repeats; and *iii.*) the entire regulatory domain including the V1-3 and C1-2 protein regions, were introduced into Rat 6 cells following subcloning into the pMV7 retroviral vector and helper-free retroviral packaging in the φ2 system. Clonal cell lines were expanded and screened by Northern and Southern blot analyses to detect clones that functionally express each of the mutant forms of PKC β_1. Further characterization by Western blotting and by assaying partially-purified PKC enzyme preparations revealed that the mutant PKC proteins have distinct migrations on SDS-polyacrylamide gels and altered enzymatic properties. Experiments are in progress to determine the effects of tumor promoters and activated oncogenes on the growth properties of these unique cell lines that express regulatory domain deletion mutants of PKC β_1.

D 450 A PHORBOL ESTER RECEPTOR/PROTEIN KINASE, nPKCη, A NEW MEMBER OF THE PROTEIN KINASE C FAMILY PREDOMINANTLY EXPRESSED IN LUNG AND SKIN, Shin-ichi Osada*,#, Keiko Mizuno#, Takaomi C. Saito#, Yoshiko Akita#, Koichi Suzuki#, Toshio Kuroki*, and Shigeo Ohno#, *Department of Cancer Cell Research Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo-108, and #Department of Molecular Biology, Tokyo Metropolitan Institute of Medical Science, Honkomagome, Bunkyo-ku, Tokyo 113, Japan.

A new member of protein kinase C (PKC) family, nPKCη(eta) was isolated from mouse epidermal cDNA library. nPKCη encodes a 78KDa protein containing a characteristic cystein-rich repeat sequence (C1 region) and a protein kinase domain sequence (C3 region). However, it lacks, like other nPKCs (δ, ε and ζ), a putative calcium binding domain (C2 region) that is seen in conventional PKCs (α, βI, βII, γ). nPKCη shows the highest sequence similarity to nPKCε (59.4% identity). Northern blot analysis showed that mRNA for nPKCη is highly expressed in the lung and skin, but, in contrast to other members of PKC family, only slightly expressed in the brain (see also Kuroki et al. in this symposium). A series of experiments using nPKCη-cDNA expression plasmid and an antiserum against its COOH-terminal peptide demonstrated that nPKCη exists at the protein level and has a phorbol ester binding activity and a kinase activity.

D 451 INVOLVEMENT OF $InsP_3$- AND Ca $^{2+}$-INDUCED Ca^{2+}-RELEASE IN THE GENERATION OF CYTOSOLIC Ca^{2+} OSCILLATIONS IN MOUSE OOCYTES, Antonio Peres, Laura Bertollini, Claudia Racca and Simona Grignani, Dip. di Fisiologia e Biochimica Generali, Univ. di Milano, Via Celoria 26, 20133 Milano Italy

Sustained pulsations in the cytosolic Ca^{2+} level are a common early response to mitogenic activation. We have investigated the mechanisms underlying Ca^{2+} oscillations by photorelease of active $InsP_3$ from its "caged" precursor in single mouse oocytes. $Ca^{2+}{}_i$ was measured with Fluo-3. Increasing the amount photoreleased $InsP_3$ (10^{-9} to 10^{-7} M) caused small, gradually increasing, Ca^{2+} changes which could, eventually, trigger a much larger, autoregenerative, Ca^{2+} transient. A further increase in the amount of active $InsP_3$ caused the appearance of a long-lasting (10 - 40 sec) plateau phase, terminated by a rapid decrease to the basal level. Often the $InsP_3$-induced Ca^{2+} transient was followed by sustained Ca^{2+} oscillations. Autoregenerative Ca^{2+} transients exhibiting "threshold" behavior and similar time course could also be generated by intracellular injection of Ca^{2+}. These results suggest that $InsP_3$-induced Ca^{2+} release may act simply as the initial trigger for a larger and autoregenerative Ca^{2+}-induced Ca^{2+} release. A theoretical model including positive feedbacks of Ca^{2+} on Ca^{2+} release and PLC activation is able to simulate most of the experimental observations.

D 452 ROLE OF c-raf-1 SER/THR PROTEIN KINASE IN GROWTH FACTOR SIGNAL TRANSDUCTION, Rapp, U.R., Kolch, W., Bruder, J., National Cancer Institute-Frederick Cancer Research and Development Center, Laboratory of Viral Carcinogenesis, Frederick, MD 21701-1201

The c-raf-1 protein kinase is activated in cells following stimulation with a variety of growth factors. These include ligands for transmembrane tyrosine kinases (PTKs) (e.g. PDGF, CSF-1, EGF, Insulin) as well as ligands for receptors with associated protein tyrosine kinases (e.g. IL-2, IL-3, GM-CSF). The mechanism for c-raf-1 recruitment varies between receptor systems but generally involves raf phosphorylation. Rapid high stoichiometry tyrosine phosphorylation was observed for receptors with associated PTKs (e.g. IL-2, IL-3, GM-CSF, CD4) whereas ligand-dependent tyrosine phosphorylation of c-raf-1 by transmembrane receptor PTKs occurred at low stoichiometry or not at all, and c-raf-1 activation required PKC-independent serine phosphorylation. A third mode of receptor coupling of c-raf-1 was seen for T cell receptor activation in 2B4 T cells that entirely depended on PKC-mediated serine phosphorylation. Given the widespread involvement of c-raf-1 in the activation of growth regulating receptors, it was important to determine whether c-raf-1 kinase activation was essential for growth induction and receptor-mediated gene regulation. We have examined this possibility in several cell systems by use of antisense constructs and a "dominant negative" c-raf-1 mutant. Both approaches have shown that c-raf-1 is essential for NIH 3T3 cell growth induced by serum or TPA. Moreover, interference with raf function blocks receptor-initiated transcription of several reporter genes.

D 454 RECONSTITUTION OF AN IP_3-INDEPENDENT HUMAN T CELL RECEPTOR-STIMULATED PLASMA MEMBRANE Ca^{2+} CHANNEL, P. M. Rosoff and C. O'Riordan, Dept. of Pediatrics, New England Medical Center, Tufts School of Medicine, Boston, MA. 02111

One of the initial events in the stimulation of resting cells is a rise in cellular Ca^{2+}. This consists of a rapid release from an intracellular storage pool and a more sustained influx across the plasma membrane. The influx of Ca^{2+} is necessary, although not sufficient, to initiate cell activation or entry into the cell cycle. The release is activated by $Ins(1,4,5)P_3$. An $InsP_3$-receptor has been cloned from brain microsomes and it can be reconstituted as an $InsP_3$-activated channel. An $InsP_3$-stimulated, low-conductance Ca^{2+} channel has also been described in human T cells. These observations suggested that $InsP_3$, produced after receptor-stimulated PIns turnover, could activate both release and influx of Ca^{2+}. However, a plasma membrane $InsP_3$ receptor/channel has yet to be purified and anti-$InsP_3$-receptor Abs do not bind to the plasma membrane. We have shown that anti-T cell antigen receptor (TCR) MoAbs rapidly stimulate an influx of Ca^{2+} in the human T cell line HPB-ALL. These cells demonstrated only influx and no internal Ca^{2+} release. This Ca^{2+} flux was membrane potential-sensitive (Ψ_p). Since these cells do not have detectable receptor-mediated increases in $InsP_3$, it suggested that a non-$InsP_3$-responsive Ca^{2+} channel may be responsible for the majority of growth factor-stimulated increases in cell Ca^{2+}. We have reconstituted such a channel from purified HPB-ALL plasma membranes which has the same characteristics as the channel *in vivo*. Vesicles were formed from detergent-solubilized plasma membranes; he optimal lipid:protein ratio was 20:1. The channel was specifically activated by anti-TCR MoAbs and was basally inactive. Ca^{2+} influx was demonstrated using either $^{45}Ca^{2+}$ or FURA2. The specific activity was 81 nmol/mg/min. An intact Ψ_p was required as the channel was inhibited by depolarization or hyperpolarization. ATP was not required for activity nor did it have a stimulatory effect. Like the channel in intact cells, the stilbene disulfonate DIDS was inhibitory. These results demonstrate the existence of a growth factor-activated Ca^{2+} channel that does not appear to require the generation of second messengers. This also implies that the TCR may be functionally coupled to a novel plasma membrane, antigen-responsive Ca^{2+} channel.

D 455 IMPROVED PURIFICATION OF CASEIN KINASE I AND GENERATION OF AN ANTI-PEPTIDE ANTIBODY, Joie Rowles, Clive Slaughter, Carolyn Moomaw, Joan Hsu, and Melanie Cobb, Department of Pharmacology, U.T. Southwestern Medical Center, Dallas, TX 75235

Casein Kinase I (CKI) has been purified to homogeneity from bovine thymus. This purification routinely recovers from 50 to 100 µg of purified enzyme from 100 g of bovine thymus. The specific activity of the purified kinase ranges from 1-2 µmoles/min/mg. Silver staining of the enzyme after SDS-PAGE shows one band of molecular weight 35,000. Tryptic peptide sequence was obtained from purified CKI. The sequences suggest that CKI may be related to the MAP2 kinases. An anti-peptide antibody was generated in rabbits. This antibody recognizes intact CKI from a number of tissues and organisms by Western blot and has been used to study the distribution of the enzyme. The antibody is also able to immunoprecipitate autophosphorylated enzyme in vitro. A 35 kDa radiolabeled band is immunoprecipitated by the antibody from ^{32}P-labeled rat fibroblasts, indicating that CKI is phosphorylated in vivo. This antibody provides a specific tool for examining the regulation, localization, and functions of this ubiquitous protein kinase.

D 456 PHOSPHATIDYLCHOLINE-SPECIFIC PHOSPHOLIPASE C IS A CENTRAL MEDIATOR OF TNF-SIGNAL TRANSDUCTION, Stefan Schütze[+], Dinko Berkovic[°], Gracia Kruppa[+], Oliver Tomsing[+], Clemens Unger[°], and Martin Krönke[+], [+]Clinical Research Group, Max-Planck-Society, and [°]Dept. of Hematology/Oncology, Medical Clinic, University of Göttingen, D-3400 Göttingen, F.R. Germany

We have recently shown that TNF induces activation and translocation of protein kinase C (PKC) in various cell lines. We here demonstrate the rapid and transient production of 1'2'diacylglycerol (DAG), a "classical" activator of PKC. While intracellular Ca^{2+} levels remained unchanged in response to TNF, the DAG production was paralleled by a rapid release of phosphorylcholine (pchol) and a concomitant decrease in phosphatidylcholine (PC). Since no changes in the choline and phosphatidic acid levels were detected, these findings strongly suggest that TNF triggers the activation of a PC-specific phospholipase C that hydrolyzes PC thereby generating both 1'2'DAG and pchol. TNF-induced PC-PLC activity as well as PKC activation were inhibited when cells were preincubated with a monoclonal anti-TNF receptor antibody (H398), demonstrating that both systems are in fact triggered by the TNF receptor. Preincubation of U937 cells with p-bromphenacylbromide (BPB) prevented TNF-induced DAG production as well as TNF-induced PKC activation. BPB at the concentration used did not inhibit TNF-stimulated $PL\text{-}A_2$ activity, suggesting that PC-PLC may be essential for TNF-stimulated PKC activation. In addition, following TNF treatment PKC activity peaked significantly earlier (i.e. at 1') than that of $PL\text{-}A_2$ (at 15'). In concert, these findings suggest that $PL\text{-}A_2$ is not involved in PKC activation. Interestingly, TNF-mediated induction of the nuclear transcription factors NF*k*B and AP1 could be inhibited by BPB, but not by a PKC inhibitor, staurosporine, indicating that TNF-induced PC-PLC may trigger additional, PKC-independent cellular signaling pathways leading to the activation of these transcription factors.

D 457 SIGNAL TRANSDUCTION FOR INITIATION OF GRANULOCYTE O_2^- PRODUCTION. G.B. Segel, T. Woodlock, S. Liang, J. Whitin, and M.A. Lichtman. University of Rochester School of Medicine and Dentistry, Rochester, NY 14642

Several intracellular second messengers including ionized calcium ($[Ca]_i$), inositol-triphosphate (IP_3), and sn-1,2-diacylglycerol (DAG) have been implicated in agonist-induced O_2^- production. In these studies, 0.1 uM fMLP stimulation generated IP3 and a peak rise in $[Ca]_i$ at 30 sec, preceding maximal O_2^- production (1.5 min) and the maximal rise in DAG mass (4 min). FMLP-induced O_2^- production was inhibited by pertussis toxin. In contrast, cytochalasin-B primed, Con A treated neutrophils exhibited a peak rise in $[Ca]_i$ but <u>not</u> IP_3 preceding O_2^- production, and pertussis toxin did not inhibit O_2^- production. Chelation of external calcium with EGTA inhibited the fMLP-induced increment in $[Ca]_i$ and O_2^- production by 75% and 50% respectively, and completely ablated the responses to cytochalasin B/Con A, suggesting a role for extracellular as well as intracellular calcium in the respiratory burst. However, three types of experiments indicate that the increase in $[Ca]_i$ is neither sufficient, nor always required, for O_2^- production. First, treatment with ionomycin resulted in a marked increase in $[Ca]_i$ but did not cause O_2^- production. Second, pertussis toxin inhibited both fMLP-induced IP_3 generation and O_2^- production but did not inhibit the rise in $[Ca]_i$. Third, following neutrophil priming with 2 uM dioctanoylglycerol (diC_8), maximal O_2^- production occurred in response to 0.015 uM (low concentration) fMLP or Con A without a rise in $[Ca]_i$, and diC_8/fMLP-induced O_2^- production was inhibited by staurosporin (kinase C inhibitor) and <u>not</u> by EGTA. Taken together, these data suggest that: 1) regulation of neutrophil $[Ca]_i$ involves mechanisms independent of IP_3 production; 2) unlike fMLP, Con A-induced O_2^- production does not proceed through a pathway involving the pertussis toxin-sensitive G protein, and 3) an increment in $[Ca]_i$ is not strictly essential for neutrophil O_2^- production. The requirement for this $[Ca]_i$ increment may be bypassed if protein kinase C is primed, e.g. by diC_8.

D 458 COMPARISON OF CD4+ CELL POPULATIONS AND CD3 AND CD2 ACTIVATION PATHWAYS IN T CELLS FROM YOUNG AND ELDERLY HUMANS. Lijun Song, James E. Nagel, Francis J. Chrest, Gary D. Collins & William H. Adler, Clinical Immunology Section, Gerontology Research Center, National Institute on Aging, NIH, 4940 Eastern Avenue, Baltimore, MD 21224
The distribution of $CD4^+$ cell subpopulations in T cell recovery column purified human T cells was examined using two color flow cytometry. No significant age differences were noted in the percentage representation of total CD4+ T cells (63 vs 57%). Antigen naive CD4+CD45R+ T cells decreased significantly (41 vs 24%; $p < 0.01$) in individuals ≥ 60 years of age, while CD4+CD45R- memory T cells increased (22 vs 33%; $p < 0.01$) in this same age group. Total CD45R+ T cells were decreased (63 vs 47%; $p < 0.01$) in the elderly. The ability of purified T cells from young and elderly donors to be activated by cross-linked anti-CD3 and soluble anti-CD2 monoclonal antibodies was also compared. Purified T cells from elderly humans stimulated with anti-CD3 incorporated less (69,100 vs 86,900 cpm) [^{3}H]TdR into cellular DNA than did T cells from young donors. However, purified T cells activated with anti-CD2 displayed no age-related proliferative differences. Northern blot analysis of early cell cycle gene expression by anti-CD2 activated T cells demonstrated no age differences in the levels of p55 IL-2 receptor or *c-myc* specific mRNA expression. In contrast, T cells from elderly individuals activated with anti-CD3 showed significant decreases in both p55 IL-2R and *c-myc* mRNA expression. T cell receptor β chain mRNA expression did not differ in either anti-CD3 or anti-CD2 activated cells. In addition to decreased CD4+CD45R+ naive T cells, the discordance in proliferative ability and specific mRNA expression between T cells from elderly donors activated with anti-CD3 or anti-CD2 provides additional evidence for a multifactorial causation of age-related T cell proliferative defects.

D 459 CHARACTERIZATION OF MEDIATORS IN THE GROWTH FACTOR-INDUCED RELEASE OF BOUND GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) FROM PERMEABILIZED RAT KIDNEY CELLS. Robert C. Stanton and Lewis C. Cantley. Renal Division, Joslin Diabetes Center and New England Deaconess Hosp., Harvard Medical School; and Physiology Department, Tufts University. Boston, Ma.
We have previously shown that EGF and PDGF stimulate the rate-limiting enzyme of the hexose monophosphate shunt, G6PD. This stimulation apparently involves release of G6PD from a binding site in the cytosol: G6PD is not released from streptolysin O permeabilized cells until growth factor addition. Moreover, the PDGF but not EGF-induced release is mediated by a pertussis toxin sensitive G-protein. The mechanism by which G6PD is released was further characterized. Since EGF and PDGF receptors have intrinsic protein-tyrosine kinase activity, a role for tyrosine phosphorylation was studied. Vanadate (200 μM), a phosphotyrosine-phosphatase inhibitor, caused a release of 7% of total G6PD, and potentiated the growth factor effects. No evidence for phosphorylation of G6PD was seen as determined by the inability to find G6PD activity in anti-phosphotyrosine antibody immunoprecipitates of growth factor-treated cells; and by the inability to find a 62 kD band (M.W. of G6PD monomer) in Western Blots from anti-phosphotyrosine antibody immunoprecipitates (and probing with anti-G6PD). Phorbol myristate acetate (PMA, 100nM), an activator of protein kinase C (PKC), alone did not stimulate release of G6PD. Yet PDGF (1 nM) and PMA together, but not EGF (10 nM) and PMA, caused a significantly greater release of total G6PD (17 %) than PDGF alone (11 %). Finally, since PDGF and EGF appear to be stimulating G6PD release via different pathways the combination of EGF and PDGF was tested. The results show that EGF and PDGF together stimulated a greater release of G6PD than either separately. These results suggest that: (1) protein phosphorylation, although not directly on G6PD, is involved in the release of G6PD; (2) PKC activation can potentiate the PDGF, but not the EGF effect; and (3) EGF and PDGF stimulate release of G6PD via different mechanisms.

D 460 CHARACTERIZATION OF PKC ISOFORMS IN AN ESTABLISHED SCLC CELL LINE: REGULATION OF PKC ε AND ITS CATALYTIC FRAGMENT, B. Strulovici, E. Oto, I. Issakani and G. Baxter, Dept. of Biochemistry, ICDB, Syntex, Palo Alto, CA. 94304.
In this study we examined the abundance, distribution and regulation of protein kinase C (PKC) isoforms in a small cell lung cancer (SCLC) cell line. Using affinity purified anti-PKC antisera, we determined that PKC ε is the major PKC isoform in the SCLC cell line N417D. PKCα and δ were present to a lesser extent, while the β and γ isoforms were undetectable. There was a 10 fold amplification of immunoreactive PKCε in the N417D cell line in comparison with other established SCLC cell lines (N69, H82 and 9080) or lung epithelial cells. Subcellular fractionation revealed that PKCε in this SCLC cell line has an unusual distribution, with 85% being in particulate form. Western blot analysis with the PKCε antiserum revealed the presence of an additional immunoreactive protein which migrated with a 40-kDa molecular mass in sodium dodecyl sulphate-polyacrylamide gel electrophoresis and showed charge heterogeneity (pI 5.1-5.6). Cytosolic extracts were fractionated by Mono Q fast protein liquid chromatography. Five peaks were revealed by using the synthetic peptide "pep-ε" ERMRPRKRQGSVRRRV as a substrate. The partially purified 40-kDa immunoreactive protein exhibited kinase activity in the absence of the PKC allosteric activators phosphatidylserine (PS) and dioctanoylglycerol (diC8), and was inhibited by 10^{-8}M staurosporine but not by PKI or sphingosine. These results indicate that the 40-kDa protein that reacts with the PKCε antiserum is the catalytic domain of PKCε. 30 min treatment of the cells with either TPA, 5×10^{-8}M or gastrin releasing peptide (GRP), a known SCLC mitogen, induced cfos mRNA expression. Prolonged presence of these agents in culture (up to 8hr) enhanced significantly the levels of both PKCε and its catalytic fragment, in a cyclohexamide-sensitive fashion. The present findings thus suggest :1) that PKCε is overexpressed in an SCLC cell line, 2) that the intact enzyme and its catalytic domain are constitutively active in these cells and 3) that their abundance and distribution are regulated by GRP and TPA possibly via a common mechanism.

D 461 **INTRACELLULAR MECHANISMS OF INTERFERON ACTION.** **Y.H. Tan, Xiao Yan, Yap W.H., Menon, D., Kowalski, S. and Guy, G.R. Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Cres. Singapore 0511.**

In cells treated with interferons rapid and transient changes in lipid metabolism occur in the first seconds/minutes as well as some long-term effects. Diacylglycerol (DAG) concentration is increased within seconds in cells treated with interferon α/β. This increase in DAG is likely to be associated with activation of protein kinase C (PKC) as the PKC inhibitor H7 can interfere with the antiviral action of interferon. Both γ- and ß-interferons also induce the rapid phosphorylation of proteins in cultured human cells. The pattern of phosphorylation of the two interferon types has some overlap which suggests they share some common pathways. In contrast to the short term changes in lipid metabolism, interferon α/β induces changes in the arachidonic acid metabolism of human fibroblasts from 5 to 20 hours after treatment. Arachidonic acid can inhibit the anti-viral action of interferon by downregulating interferon receptors.

It appears that interferons can initiate intracellular signals by inducing phosphorylation of key substrates which may include latent transcriptional factors. Later changes in lipid metabolism could augment or inhibit the initiating signal. Arachidonic acid and its metabolites are thought to be part of this feed-back or feed-forward mechanism.

D 462 **CHARACTERIZATION OF A TPA-DEPENDENT JURKAT VARIANT THAT EXPRESSES A HIGH LEVEL OF IL-2R BUT HAS LOST CD45 EXPRESSION,** **Kam-Meng Tchou-Wong and I. Bernard Weinstein, Institute of Cancer Research, Columbia University, New York, NY 10032**

In studying the role of protein kinase C (PKC) in T cell signaling, we have used 12-O-tetradecanoyl-phorbol-13-acetate (TPA) as an activator of PKC in the Jurkat T leukemia line. We have thus isolated variants of Jurkat cells that are refractory to the growth-inhibitory effects of TPA. In contrast to the commonly isolated TPA-resistant lines, a rare variant (P13-5) has been isolated that is actually dependent on TPA for growth. In the absence of TPA, the P13-5 variant becomes growth-arrested in G_1. Furthermore, in the continuous presence of TPA, P13-5 cells express a sustained, high level of the alpha subunit of interleukin-2 receptor (IL-2R). Whereas the parental Jurkat and other TPA-resistant cells, which are independent of TPA for growth, express high levels of CD45 (a membrane-associated protein tyrosine phosphatase), the P13-5 variant has lost surface expression of CD45. It remains to be determined whether the loss of CD45 is the result or the cause of the acquisition of TPA-dependent growth. This variant will prove to be useful for elucidating the role of protein kinases and phosphatases in the control of T cell activation and proliferation.

D 463 **CD69, A SIGNAL TRANSDUCING SURFACE HOMODIMER FOUND ON ACTIVATED LYMPHOCYTES, IS FUNCTIONALLY EXPRESSED ON RESTING PLATELETS.**
Roberto Testi, Fabio Pulcinelli, Luigi Frati, Pier Paolo Gazzaniga and Angela Santoni, Department of Experimental Medicine, University of L'Aquila and Department of Experimental Medicine, University of Rome, Italy.

PKC activation by signals generated at the level of the TCR/CD3 complex on T lymphocytes results in the rapid expression of CD69, a 28-32 kD disulphide-linked homodimer. Both chains of the dimer appear phosphorylated on at least one Tyr residue. TCR/CD3-mediated CD69 induction is RNA and protein synthesis dependent, but not sensitive to CsA. Specific crosslinking by MoAbs of CD69 molecules on T cells induces prolonged Ca^{++} influx and, together with a simultaneous stimulation of PKC, IL-2 gene expression and eventually cell division. CD69-mediated IL-2 gene expression is CsA sensitive and dependent on the surface expression of the TCR/CD3 complex on T cells. A molecule which antigenically, biochemically and functionally resembles CD69 was found on resting platelets. CD69 crosslinking by MoAbs on platelets results in Ca^{++} influx, activation of the cycloxigenase pathway, possibly by PLA2 activation, with production and secretion of PGE2 and TXA2, degranulation with ATP release, and finally platelet aggregation. These data show for the first time that lymphocytes and platelets share signal tranducing molecules, and suggest a more general role for CD69 in cellular activation.

D 464 "32P-PIP3 AND 32P-PI(3,4)P2 ARE ELEVATED IN ELECTROPORATED NEUTROPHILS AND HL60 CELLS" Alexis Traynor-Kaplan. UCSD Medical Center, H811D2, UCSD, San Diego, CA 92103.

PI Kinase activity has been associated with growth factor receptors and increases in novel phosphoinositides (PIPs) have been identified in neutrophils stimulated with chemotactic agonists. These lipid products are short lived, however, and neither the factors regulating their metabolism nor their role in cell function have been elucidated. The experiments described here addressed two goals: 1) to generate large quantities of novel PIPs to further investigate their properties; 2) to determine if electroporated cells were suitable for investigation of their intracellular role and metabolism. Neutrophils and HL60 cells were electroporated in the presence of 32P-ATP. After 2 min. the cells were added to chloroform / methanol and the lipids extracted. Novel PIPs were separated and identified by TLC and HPLC of deacylated lipids. 99% of the radioactivity in phospholipids from electroporated PMNs or HL60 cells were found in either PA, PI, PIP, PI(34)P2, PI(45)P2, or PIP3. The relative amount of 32P incorporated into the individual phospholipids could be modulated by varying Ca^{++}. Higher concentrations of Ca^{++} favored the formation of 32P-PIP3 whereas the presence of the tyrosine kinase inhibitor, tyrphostin slightly inhibited the formation of 32P-PIP3. Adenosine also inhibited 32P-PIP3 formation relative to other lipids. While GTPgammaS and PMA stimulated the production of 32P-PA, relative amounts of 32P-PIP3, PIP2 and PIP were diminished. In conclusion, it should be possible to use electroporated cells to generate novel PIPs. Furthermore, this protocol may be useful in evaluating the contribution of tyrosine kinase to novel phosphoinositide production although it does not appear suitable for studying the involvement of G-proteins in 32P-PIP3 production.

D 465 CONSTITUTIVE PRODUCTION OF A PUTATIVE GROWTH FACTOR BY A CELL LINE WHICH OVEREXPRESS PKC$_{\beta I}$, Marius Ueffing, Sarah N. Guadagno, Sadayori Hoshina, Jean Cadet, and I. Bernard Weinstein, Institute of Cancer Research, Columbia *University, New York, NY 10032.*

In previous studies we used a Rat-6 cell line which overexpresses the βI isoform of PKC (R6-PKC3) to demonstrate that alterations in cellular levels of PKC can markedly influence the response of cells to specific growth factors. We found that certain agents when tested alone in serum free medium (EGF, PDGF, TPA, telocidin, and OAG) stimulate DNA synthesis in quiescent R6-PKC3 cells but not in the corresponding control cell line R6-C1 (Hoshina, Ueffing *et. al*, J. Cell Phys., in press). We also found that conditioned media (32 hr medium supernatants) from the R6-PKC3 cells can act alone or in cooperation with EGF to stimulate DNA synthesis in the R6-C1 cells. We have now isolated a growth promoting factor from these supernatants that stimulates DNA synthesis in fibroblasts. This factor can also induce proliferation and subsequent differentiation of LAN-5 neuroblastoma cells. PAGE-analysis of proteins from cell supernatants indicate that the secretion of this factor is highly enhanced in R6-PKC3 cells when compared to the very low levels seen in R6-C1 supernatants. These studies show that elevated levels of PKC can lead to disturbances in growth control and the production of a putative autocrine growth factor which thereby reduces the dependence of these cells on exogenous growth factors.

D 466 cAMP DEPENDENT MODULATION OF HUMAN B CELL RESPONSE TO IL.2 AND IL.4. Aimé Vazquez, Marie-Thérèse Auffredou, Pierre Galanaud and Gérald Leca. INSERM U 131, 32 rue des Carnets, 92140 Clamart, France.

The second messager cAMP is a modulator of cellular growth possesing both inhibitory and stimulatory properties. In this report, we show that IL.2 and IL.4 dependent DNA synthesis of anti-μ activated human B cells is modulated in opposite ways by agents increasing cAMP internal level. bt2cAMP and forskolin, although devoid of proliferating properties by themselves, decrease the IL.2 driven proliferation whereas IL.4 mediated DNA synthesis is slightly increased. cAMP and IL.4 inhibit the IL.2 dependent proliferation with a similar pattern of reactivity. Both IL.4 and forskolin need to be present during the first 48 h of culture, and preactivation for 16 h with forskolin and IL.4 does not prevent further B cell response to IL.2, suggesting that cAMP and IL.4 directly interact with IL.2 signalling. Furthermore we provide evidence that in contrast to IL.2, IL.4 signalling leading to B cell proliferation in PKC independent. In addition we show that the cAMP-dependent proteine kinase inhibitor H8 reverses the IL.4 inhibitory effect on IL.2 driven proliferation. Our data suggest that IL.4 inhibitory signal to IL.2 driven proliferation involves cAMP-dependent protein kinase activation. The mechanism of this inhibition will be discussed.

Growth Factor Signal Transduction

Growth Control: Positive and Negative

D 500 CYTOREDUCTIVE THERAPY OF MULTIDRUG RESISTANT HEPATOCELLULAR CARCINOMA: NEGATIVE REGULATION OF GROWTH USING COMBINATION DIFFERENTIATION THERAPY, Edwin W. Ades and James M. Pruckler, Biological Products Branch, Centers for Disease Control, Atlanta, GA 30333

In vitro studies of the mouse erythroleukemia cell system have identified at least 300 agents capable of inducing differentiation by mechanisms that remain to be elucidated. We have recently begun to examine recombinant cytokines as possible agents in inducing differentiation of tumor cells, specifically, malignant cells resistant to cytotoxic drugs. One such cytokine, transforming growth factor - beta (TGF-B), is a multi-functional peptide that exists in at least 5 different isoforms in vertebrate species. Recently, there has been a great deal of interest in the role of TGF-beta as an important multi-functional growth regulator; inducing cells of mesenchymal origin to divide while inhibiting the growth of nontransformed epithelial cells.

In this study, we combine the effects of the differentiation agent hexamethylene bisacetamide (HMBA) and the inhibiting effects of TGF-B on a multi-drug resistant human liver hepatocellular carcinoma and demonstrate the cytoreductive synergistic interaction of these two inducers of differentiation. These data support the concept of terminal cell division or programmed cell death.

D 501 INDUCTION OF EARLY RESPONSE GENES IN GLIAL CELLS BY HORMONES AND GROWTH FACTORS: INTERACTING PATHWAYS, Alaric T. Arenander, Janet Cheng and Jean de Vellis, Mental Retardation Research Center, Department of Anatomy and Cell Biology and the Brain Reseach Institute, UCLA, LA, CA, 90024

The proliferation and differentiation of glial cells are regulated by a wide spectrum of hormones and growth factors. In order to study the early nuclear events which accompany the induction of biochemical and morphological change in glial phenotype during development, the pattern of expression of a number of early response genes (ERGs) were examined in cultures of rat neocortical astrocytes and C6 glioma cells. Cultures were treated with a variety of agents, either alone or in combination, including TPA, forskolin, bFGF, EGF, retinoic acid, hydrocortisone, insulin, IGF-1, and tri-iodothyronine. Expression of ERG mRNAs was examined by Northern blot analysis and in situ hybridization. Results suggest considerable interaction exists among intracellular pathways activated by various ligands as seen in altered kinetics and levels of ERG mRNA expression. Since ERGs participate in the transduction of environmental signals, the differential induction of ERGs by various growth factors may act as a 'filter' determining the stage-specific responsiveness of glial cells during development. (Supported by DOE and NIMHD Grant 06576)

D 502 STIMULATION OF BONE FORMATION AND OSTEOGENIC CELL ACTIVITY BY PEPTIDE ISOLATED FROM REGENERATING BONE MARROW, Itai Bab, Dan Gazit, Andras Muhlrad, Arye Shteyer, and Michael Chorev, Hebrew University-Hadassah Schools of Dental Medicine and Medicine, Jerusalem, Israel.

Postablation healing marrow produces growth promoting activity to osteogenic cells. This activity presumably mediates the local and systemic osteogenic responses seen during marrow regeneration. Now, an activity derived from the regenerating marrow has been purified to homogeneity. Amino acid sequencing revealed a 14-residue osteogenic growth peptide (OGP) homologous to the carboxy terminus of histone H4. In addition, OGP shares a 5 amino acid motif with the T-cell receptor ß-chain V-region and Bacillus subtilis outB locus. An identical synthetic peptide (sOGP) stimulated the proliferation and alkaline phosphatase activity of rat osteosarcoma cells (ROS 17/2.8) at 10^{-13}-10^{-8} M. These effects were absent following iodination of Tyr^{10} and markedly reduced consequent to modifications of the amino or carboxy terminus, suggesting the occurrence of a highly specific OGP receptor. When injected i.v. to adult rats sOGP stimulated osteogenesis at 1-10 ng/rat/day for 8 days. This enhancement in bone formation was unaccompanied by increased resorption and was similar to the postablation systemic osteogenic response. These data substantiates, for the first time, a role for small peptides in the regulation of osteogenesis.

D 503 ACTIVATION OF THE *ras*-RESPONSIVE NUCLEAR FACTOR, RRF1, BY A POST-TRANSLATIONAL MECHANISM, Donna M. Bortner[1], Russell D. Owen[2], Massimo Ulivi[1], and Michael C. Ostrowski[1]. [1]Department of Microbiology and Immunology, Duke University Medical Center, Durham, NC 27710; [2]Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709.
Activation of transcription by *ras* oncogenes has been shown to require a 120 kDa nuclear protein, RRF1, present in *ras*-transformed cells. Studies with cells transformed by the v-*fms* oncogene indicate the presence of RRF1 in these cells also. Transcription of the *ras*-responsive gene, NVL-3, is upregulated in v-*fms*-transformed cells, and a 120 kDa nuclear protein present in these cells binds to the *ras*-responsive element of NVL-3. In non-transformed cell lines that stably express the c-*fms* proto-oncogene, which encodes the CSF-1 receptor, transcription of the NVL-3 gene is detected at only a low level. Treatment of these cells with CSF-1, which causes cellular transformation, also causes stimulation of transcription of the NVL-3 gene. In addition, the presence of RRF1, as determined by gel retention assays, is apparent in nuclear extracts from these cells only after CSF-1 treatment. The increase in NVL-3 transcription and activation of RRF1 DNA binding activity are observed within 15 minutes after CSF-1 treatment. A similar response is observed when CSF-1 treatment is performed in the presence of cycloheximide, indicating RRF1 may be activated by a post-translational modification. Current efforts are aimed at analyzing the mechanism of RRF1 activation.

D 504 C-JUN VERSES V-JUN: A FUNCTIONAL COMPARISON OF *in vitro* TARGETS. Timothy J. Bos, Department of Microbiology and Immunology, Eastern Virginia Medical School, P.O. Box 1980, Norfolk, Virginia 23501.
Viral Jun was first discovered as the oncogenic effector of Avian Sarcoma Virus 17. It's cellular counterpart, c-Jun, has been extensively characterized and found to be a major component of the AP-1 transcription complex. C-Jun readily dimerizes with the product of another proto-oncogene, c-Fos, through a structural motif termed the leucine zipper. C-Jun and v-Jun exhibit some important structural and functional differences. Both v-Jun and c-Jun have been shown to bind specifically to several variations of the AP-1 consensus sequence. Both have been shown to form a complex with c-Fos and both have been shown to activate transcription of AP-1 - reporter plasmids. In addition, overexpression of c-Jun has been shown to induce cell transformation in several cell types both as a single gene (primary CEF or Rat 1 cells) or in cooperation with an activated ras gene (primary rat cells). The mechanism(s) by which Jun overexpression leads to cell transformation is not clear. Recent studies in CEF have indicated that the efficiency by which c-Jun induces cell transformation is low when compared to viral Jun. The difference in oncogenic activity between c-Jun and v-Jun has been mapped to structural changes in v-Jun in both coding and non-coding sequences (Bos et al.,1990. Genes and Development, in press). Interestingly, a quantitative difference in the transcriptional activation capability between v-Jun and c-Jun has also been noted and mapped to the same structural region implicated in transformation (Bohmann and Tjian. 1989. Cell 59, 709-717). This raises the possibility that c-Jun exists in transcriptionally active and inactive states within the cell and that viral Jun may represent a constitutively active form of c-Jun. In addition, differences in transcriptional activity may result from changes in regulatory specificity between v-Jun and c-Jun. For example, v-Jun may activate targets that c-Jun represses. With this in mind we have initiated a comparison of the regulatory specificities of viral and cellular Jun on a number of *in vitro* targets. The results of this study will be discussed.

D 505 Suppression of Oncogenic Transformation by a Phosphotyrosine Phosphatase
Sheryl Brown-Shimer, David E. Hill, and Arthur Bruskin.
Applied bioTechnology, Inc. 80 Rogers St. Cambridge, MA 02142. 617-492-7289.
Phosphotyrosine phosphatases (PTPases) have been implicated in the regulation of signal transduction mediated by receptor tyrosine kinases. In order to test the hypothesis that PTPases could function as growth suppressors or anti-oncogenes, we have recently cloned and mapped the PTP1B gene encoding a low molecular weight, cytoplasmic PTPase[1]. To analyze the functional role of PTPases in growth control and signal transduction, NIH 3T3 cells have been transfected with either a plasmid expressing PTPase from a retroviral LTR or a plasmid with PTPase under the control of the inducible metallothionein promoter. PTPase-expressing transfectants have been identified by western blotting. While LTR-driven PTPase expression is constitutive, PTPase expression from the metallothionein promoter is induced upon addition of heavy metals to the medium. Control 3T3 cells and PTPase-expressing cells have been infected with retrovirus expressing an oncogenic form of human neu. Presence of human neu in infected cells was confirmed by ELISA assay and neu kinase activity was shown by *in vitro* autophosphorylation assays following immunoprecipitation with anti-neu monoclonal antibodies. Whereas control cells are transformed by neu, cells expressing relatively high levels of PTPase from an LTR do not appear to be transformed as measured by focus formation or colony formation in soft agar; tumorigenicity studies are presently underway. Current efforts are directed toward identifying which phosphotyrosine-containing proteins are the targets of PTPase action in cells co-expressing an oncogenic tyrosine kinase.
1. Brown-Shimer, et al (1990) Proc. Natl. Acad. Sci. USA 87, 5148-5152.

D 506 CLONING OF A cdc2+/CDC28-HOMOLOGOUS GENE FROM *Arabidopsis thaliana*, John L. Celenza and Gerald R. Fink, The Whitehead Institute for Biomedical Research, Cambridge, MA 02142

We are interested in how plants regulate their growth, and as a first step we have cloned a cdc2+/CDC28 homolog from the dicotyledonous plant *Arabidopsis thaliana*. Degenerate oligonucleotides spanning conserved regions of the cdc2+/CDC28 family of protein kinases were used in a polymerase chain reaction in order to isolate homologous sequences from Arabidopsis genomic DNA. Clones that were similar to the cdc2+/CDC28 family were used to probe an Arabidopsis cDNA library and a complete cDNA was identified that could encode a 34 kd protein that is 65% identical to the CDC28 protein of *S. cerevisiae*. The high level of shared sequence identity is conserved throughout the entire sequence, and the Arabidopsis protein contains the VPSTAIR sequence unique to the cdc2+/CDC28 family of protein kinases. We have also found that when the Arabidopsis gene is expressed in yeast it can complement a temperature-sensitive *cdc28* mutation. In order detect the Arabidopsis protein we have epitope tagged the protein with an influenza hemagglutinin antigen and have identified the protein expressed in yeast on an immunoblot. We are currently making transgenic Arabidopsis that will carry the epitope tagged protein. We will then be able to monitor the synthesis and activity of the protein kinase throughout the plant life cycle and begin to determine what factors control plant cell division.

D 507 PDGF ISOFORM SPECIFIC DIFFERENCES IN COMPETENCE FORMATION
Steven R. Coats, A.F. Candia and W.J. Pledger, Vanderbilt University, Department of Cell Biology, Nashville, TN 37232

Cell cycle competence induced by platelet-derived growth factor (PDGF) has been shown to be dependent upon the specific PDGF isoform present. Experiments performed in our laboratory demonstrate that the PDGF isoform, BB, is capable of inducing competence at a lower concentration and with a shorter exposure time than PDGF-AA.

Our experiments indicate that PDGF-AA (25 ng/ml) must be present for a period of at least 16 hours to allow cell to traverse the cell cycle. Using the same concentration of PDGF-BB the fibroblasts are competent to progress through the cell cycle after a pretreatment period of 2-4 hours. The timing of cells entering S phase is identical when using different isoforms of PDGF to induce DNA synthesis. Presently we are studying the different mechanisms used by PDGF-AA versus PDGF-BB to initiate cellular proliferation.

To determine if differences in downregulation and subsequent return of α versus β PDGF receptors is responsible for the longer time required for PDGF-AA induction of DNA synthesis, PDGF binding experiments were performed. PDGF binding data indicates that the return of PDGF α receptors starts to occur within 30 minutes after downregulation of α receptors by PDGF-AA treatment for 1 hour at 37°C. Within 4 hours after PDGF treatment the level of PDGF-AA binding was equivalent to the binding present on cells prior to PDGF exposure. The return of α receptors on the cell surface is dependent upon protein synthesis. Even though the number of α receptors present on Balb/c3T3 fibroblasts is less than β receptors, we have observed an equivalent induction of c-fos mRNA in fibroblasts stimulated with either PDGF-AA or PDGF-BB. This indicates that an early event associated with PDGF stimulation is present in fibroblasts in which only the α receptor has been activated.

D 508 EXPRESSION AND CHARACTERIZATION OF A BIOLOGICALLY ACTIVE HUMAN PLATELET-DERIVED GROWTH FACTOR B CHAIN IN ESCHERICHIA COLI, B. L. Dalie, D. Alexander, M. Shanahan, T. Hesson*, A. Mannarino*, M. Cable*, H. V. Le*, P. P. Trotta*, P. J. Zavodny, R. Greenberg and S. K. Narula. Departments of Biotechnology/Molecular Biology and Biotechnology/Biochemistry*, Schering-Plough Research, Bloomfield, New Jersey, 07003

Platelet-derived growth factor (PDGF) is a major mitogen that stimulates the proliferation of mesenchymally derived cells in vitro. PDGF is a disulfide-bonded dimer of two related polypeptide chains, termed A and B. Type AA, BB, and AB PDGF dimers have been found in several cell types; all three dimeric forms of PDGF have been shown to be biologically active. PDGF exerts its mitogenic effect by binding with high affinity to a specific cell-surface receptor, eliciting a pleiotropic response that ultimately leads to enhanced cellular proliferation. Recent evidence has implicated PDGF in the intimal proliferation associated with atherosclerosis. A detailed analysis of PDGF structure will aid in elucidating the nature of the interactions that govern the binding and activation of its specific receptor; efficient expression of biologically active PDGF in microorganisms would facilitate further studies on PDGF aimed at defining its role in cellular proliferation in normal and diseased states. We have constructed an expression plasmid that directs the synthesis of intact, mature hPDGF-B in E.coli. In our system, hPDGF-B is expressed as an insoluble, intracellular aggregate and can be obtained in a highly homogeneous form from inclusion body preparations. Sufficient quantities of purified material can be prepared from this system to support biochemical and biological analysis. Automated Edman degradation of the recombinant hPDGF-B has verified that the N-terminal sequence is identical to that predicted from the cDNA. The dimeric recombinant hPDGF-B is competitive in a receptor-binding assay and is biologically active in an in vitro assay for mitogenesis.

D 509 **PDGF MOBILIZES MULTIPLE CALCIUM POOLS IN 10T1/2 FIBROBLASTS.** Mark Estacion & Lawrence Mordan, Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI 96813.
Fura-2 fluorescence was used to monitor cytoplasmic calcium levels while exposing the CH3/10T1/2 fibroblasts to mitogenic amounts of PDGF. The PDGF-induced calcium transient shows both peak and sustained components. By manipulating the external calcium concentration at defined times, either before or during PDGF stimulation, multiple pools of the calcium response could be resolved. Reduction of the external calcium by addition of 10 mM EGTA shortly before PDGF stimulation caused only a slight decrease in the PDGF-induced calcium signal. This is a manipulation which reduces by more than 80% the mobilization of the IP_3-sensitive Ca^{2+}-pool. In contrast, changing to low external calcium for 60 to 90 minutes prior to PDGF stimulation results in a nearly absent signal. Restoring these low calcium equilibrated cells to normal external calcium levels reconstituted the PDGF-induced calcium transient with multiple kinetics, the sustained component recovering faster than the peak component. These results suggest a model with at least two intracellular and at least one extracellular Ca^{2+}-pool sensitive to PDGF stimulation. The relative roles of these pools in the observed reduction of mitogenic stimulation by PDGF following retinoid treatment, or by culture in low extracellular calcium levels, is being pursued.

D 510 The 85-kDa TGFß receptor subtype as a mediator of TGFß1 action in endothelial cells
V. Fafeur, B. O'Hara and P. Böhlen
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Two transforming growth factor-beta (TGFß) receptor subtypes of molecular weights 85-kDa and 65-kDa were identified by affinity labelling techniques in bovine endothelial cells. While it has been recently proposed that the 65-kDa receptor subtype mediates TGFß action (Boyd and Massagué, J. Biol. Chem, 264:2272, 1989), the function of the 85-kDa receptor subtype is unknown. By chemical mutagenesis, we isolated a clone of endothelial cells resistant to growth inhibition by TGFß1. We examined the TGFß receptors and found that the molecular weight of the 85-kDa receptor subtype was modified, while that of the 65-kDa receptor subtype was unchanged. In addition, we recently reported that in normal endothelial cells, basic fibroblast growth factor (bFGF) can decrease the growth inhibitory effect of TGFß1 and this effect is accompanied by a down-regulation of only the 85-kDa TGFß receptor subtype (Fafeur et al, Growth Factors, in Press). The fact that TGFß1 was found to be less effective when the 85-kDa TGFß receptor subtype was either modified in the mutant cell or down-regulated by bFGF suggest that this subtype at least in part mediates TGFß1 action.

D 511 **TYROSINE PHOSPHORYLATION OF P34 cdc2 IN VITRO BY AN ASSOCIATED TYROSINE KINASE: CORRELATION WITH FUNCTION**, Douglas K. Ferris[1], David J. Kelvin[2], Gretchen A. White[1], Chou-Chi Li[1] and Dan L. Longo[2], [1]BCDP, Program Resources, Inc., NCI-FCRDC, Frederick, MD 21702, [2]BRMP, NCI-FCRDC, Frederick, MD 21702
P34 cdc2, a 34 KDa serine/threonine kinase first identified in yeast, is the catalytic subunit of maturation promoting factor in frog oocytes and is also involved in regulating mitosis in mammalian cells. It has previously been shown that p34 cdc2 is phosphorylated on tyrosine _in vivo_ by an unidentified tyrosine kinase in a cell cycle dependent manner. Rapid tyrosine dephosphorylation occurs in late G2/M before p34 cdc2 histone H1 kinase activity is maximal. It is thought that tyrosine phosphorylation negatively regulates the activity of p34 cdc2 in some fashion, although tyrosine dephosphorylation _in vitro_ has not been shown to be sufficient to activate p34 cdc2 histone kinase activity. Using gentle lysis and immunoprecipitation conditions we have found that p34 cdc2 is associated with a tyrosine kinase that phosphorylates it _in vitro_. _In vitro_ tyrosine phosphorylation of p34 cdc2 by the associated tyrosine kinase reduced p34 cdc2 histone H1 kinase activity 5-10 fold and also affected the association of p34 cdc2 with other proteins _in vitro_.

D 512 REGULATION OF INTERLEUKIN 2 DEPENDENT GROWTH RESPONSES BY GLYCOSYL-PHOSPHATIDYLINOSITOL MOLECULES, Glen N. Gaulton, Joanne C. Pratt, and Isabel Merida, University of Pennsylvania School of Medicine, Philadelphia, PA 19104

The molecular mechanism of signal transduction through the interleukin 2 (IL2) receptor remains an enigma. Glycosyl-phosphatidylinositol (GPI) lipids were investigated as one component of this process. IL2 stimulated the rapid (30 sec) loss of >50% of GPI in the IL2 dependent cell line CTLL-2. Half maximal GPI loss was detected at 40 pM IL2, coincident with the EC_{50} (20 pM) for IL2 induced proliferation of this cell line. This effect was specifically inhibited by antibodies that bind either IL2 or the IL2 receptor. The loss of GPI was mirrored by the accumulation of both polar inositolphosphoglycan (IPG) and diacylglycerol lipid fragments within cells. Increases in lipids were initially restricted to myristyldiacylglycerol, but were followed by the accumulation of myristylphosphatidic acid. These results are indicative of IL2 dependent hydrolysis of GPI in T cells. The biological relevance of this hydrolysis was demonstrated by synergism of purified IPG with IL2 in T cell proliferation responses. The inclusion of IPG (0.1uM) in determinations of IL2 dependent CTLL-2 growth shifted the EC_{50} from 20 pM to 7 pM IL2. IPG had no effect on either the number or affinity of IL2 receptors, therefore, half maximal CTLL-2 proliferation was obtained at <10% IL2 receptor occupancy. These results demonstrate that GPI lipids are an important component of the biological response to IL2.

D 513 REGULATION AND PROCESSING OF A PROTEIN THAT MEDIATES CELL DENSITY SENSING, Richard H. Gomer, Ita S. Yuen and Renu Jain, Howard Hughes Medical Institute, Department of Biochemistry and Cell Biology, Rice University, Houston, TX 77251-1892

During *Dictyostelium* development the expression of several genes is regulated in part by cell density. For instance, prestalk and prespore genes can be expressed by high-density starved cells but not by low-density starved cells. The density sensing is mediated by a secreted factor: low-density cells will express prestalk and prespore genes when exposed to starvation buffer previously conditioned by a high density of starved cells (conditioned medium). One possible explanation for this is that during development cells need to be able to sense whether they are far from an aggregation center and thus need to continue expressing aggregation-specific genes, or whether they are at or near an aggregation center and can thus begin expressing differentiation-specific genes. Fractionation of conditioned medium on Sephadex G-50 shows two size classes of conditioned medium activity. One can be purified to an 80 kD glycoprotein (Gomer et al., submitted); the partial sequence of the corresponding cDNA shows little similarity to any known protein. The other class of conditioned medium factor (CMF) is a set of 1- 5 kD polypeptides, some of which are glycosylated. These polypeptides appear to be breakdown products of the 80 kD CMF: When 80 kD CMF is allowed to sit out at room temperature, the breakdown products co-elute on G-50 and reversed phase HPLC with the small CMF molecules from whole conditioned medium. Assays of serial dilutions indicate that there is a roughly 25 fold increase in the effective specific activity of CMF upon breakdown.

Secretion of CMF begins approximately 6 hours after starvation and is enhanced by the pulses of cAMP that mediate chemotaxis. Diffusion calculations using the measured secretion rate (12 molecules/ cell/ minute) and sensitivity of cells to CMF (0.3 ng/ml) suggest that only those cells at the aggregation center will initially differentiate. The breakdown of CMF to a faster-diffusing, higher specific activity form then might allow cells further from the aggregation center to differentiate.

D 514 SEPARATE NEGATIVE CONTROLS OF CELL PROLIFERATION AND DIFFERENTIATION IN SELF-TOLERANT B-LYMPHOCYTES, Christopher C. Goodnow, Elizabeth Adams+ and Robert A. Brink+, Department of Microbiology and Immunology, Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford CA 94305. +Centenary Institute for Cancer Medicine and Cell Biology, University of Sydney, NSW 2006 Australia.

The immune system normally avoids producing antibodies to self antigens by eliminating or inactivating T and B lymphocytes with receptors which are specific for self antigens. In a transgenic mouse model, we have previously found that B lymphocytes specific for lysozyme were rendered tolerant but not eliminated in double-transgenic mice expressing lysozyme as a transgene-encoded self antigen. The tolerant state exhibited by these B lymphocytes involves two separate intrinsic changes. Firstly, IgM antigen receptors are dramatically downregulated and IgD antigen receptors, although not downregulated, are desensitized with respect to induction of B cell proliferation. Stimulating the tolerant B cells with the nonspecific mitogen LPS, to bypass the antigen receptors, allows normal proliferation but reveals a profound block in their differentiation into antibody-secreting cells. The two controls are dissociable, and both are potentially reversible in the absence of continued antigen encounter. Lysozyme, a monomeric ligand, induces these profound changes through a functionally distinct signalling mode of the B cell antigen receptor.

D 515 MAPPING THE ANCHORAGE-DEPENDENT GROWTH CONTROL WITHIN THE CELL CYCLE OF NRK FIBROBLASTS, Thomas M. Guadagno and Richard K. Assoian, College of Physicians and Surgeons, Columbia University, New York, NY 10032

We have developed a preparative suspension culture system that allows us to compare cell cycle progression of adherent and non-adherent NRK fibroblasts. Northern analysis of mRNA isolated from G0/G1 synchronized cells was used to localize the block in cell cycle progression of suspension cultures exposed to serum and EGF. Interestingly, results from these experiments showed that the kinetics and induction of c-*fos* and c-*myc* were equivalent in monolayer and suspension cultures. Nevetheless, parallel experiments examining effects on cell number and DNA synthesis showed that the suspended cells failed to divide or incorporate ^{3}H-thymidine into DNA. These results demonstrate that non-dividing NRK fibroblasts in suspension enter the cell cycle and express early G1 markers. Thus, the control mechanism preventing anchorage-independent growth of these cells can be localized to the mid-G1/S phase, prior to DNA replication. These results also define a window for analysis of sub-cellular events relating directly to TGF-ß induced transformation.

D 516 SERINE/THREONINE PROTEIN PHOSPHATASE ACTIVITY IN THE CELL CYCLE: STUDIES WITH NODULARIN, MICROCYSTIN-LR AND OKADAIC ACID. Richard E. Honkanen, Mike Dukelow, Kathy Tse and Alton L. Boynton, Cancer Research Center of Hawaii, Honolulu HI, 96813.

The importance of protein kinases in cell cycle regulation is well documented; however, by comparison the role of protein phosphatases has been relatively ignored. This is partly due to a lack of specific inhibitors that can be used to study the functions of protein phosphatases in vivo. Here we characterize the effects of nodularin and compare them to the effects of two other recently discovered protein phosphatase inhibitors, okadaic acid and microcystin-LR. Nodularin potently inhibits type 1 (PP1) and type 2A (PP2A) protein phosphatases demonstrating greater potency than microcystin-LR against PP2A and equal potency against PP1. Both nodularin and microcystin-LR are >10 fold more potent inhibitors of PP1 and PP2A than okadaic acid, and at concentrations $\leq 1\ \mu$M none of these compounds affect PP2B or PP2C. All three compounds inhibit the activity of PP2A at a 10-100 fold lower concentration than that required to inhibit the activity of PP1 enabling the use of these compounds to probe the cellular functions of PP1 and PP2A. Microinjection of microcystin-LR and okadaic acid into sea urchin eggs at a concentration that inhibits PP2A, but not PP1, indicates that PP2A is involved in the onset of pronuclear migration and nuclear envelope breakdown. In mammalian cells okadaic acid also blocks T51B cells at metaphase.

D 517 TRANSFORMING GROWTH FACTOR ß1 INCREASES IgA ISOTYPE SWITCHING AT THE CLONAL LEVEL, Martin F. Kagnoff, Pyeung-Hyeun Kim, Department of Medicine, University of California, San Diego, La Jolla, CA 92093

TGFß1 has important effects on the expression of the IgA isotype. TGFß1 alone, or in combination with interleukin 5 (IL-5) or IL-2 increases IgA secretion by populations of lipopolysaccharide (LPS) activated surface IgA$^-$ (sIgA$^-$) spleen B cells, while concurrently decreasing IgM and IgG secretion. The present study demonstrates the activity of TGFß1 as an IgA isotype switch factor at the clonal level. Stimulation of LPS-activated surface IgA$^-$ spleen B cell populations with TGFß1 resulted in a significant increase in total numbers of IgA secreting cells, and this increase ultimately was paralleled by an increase in total IgA secretion. By limiting dilution analysis, TGFß1 was shown to increase the frequency of IgA secreting B cell clones by approximately 20-fold. This was not accompanied by increased numbers of IgA secreting cells per clone. Other cell cycle inhibitors also selectively increased numbers of IgA secreting cells and total IgA secretion among populations of LPS-activated sIgA$^-$ spleen B cells, although to a lesser extent than TGFß1. This suggests the IgA enhancing activity of TGFß1 may, in part, be related to its ability to inhibit cell growth.

This work was supported by NIH Grant #DK35108.

D 518 MOLECULAR CLONING AND CHARACTERIZATION OF A MOUSE HOLOGUE OF THE FISSION YEAST CELL CYCLE CONTROL GENE *cdc25+*, Akira Kakizuka, Merl Hoekstra*, Ronald M. Evans, Howard Hughes Medical Institut, Gene Expression Lab, *Molecular Biology and Virology Lab, The Salk Institute, P.O. Box 85800, San Diego, CA 92186

A diversity of extracellular signal molecules, including growth factors and morphogens, have been identified which control mammalian cell proliferation and development. Although the intracellular targets of these signals are still obscure, they must change the expression patterns of genes crucial for growth control and differentiation. One plausible candidate for these targets could be the *cdc25+* gene, which was originally isolated from *S. pombe* as a rate limiting mitotic inducer and then isolated from *D. melanogaster* as a gene required for regulation of the embryonic cell cycle. We have isolated a cDNA clone encoding a mouse homologue of *cdc25+* from an embryonic teratocarcinoma stem cell cDNA library, using low stringency hybridization. The predicted amino acid sequence of the mouse homologue shares 45% and 50% identity in the C terminal region with the *S. pombe* and *D. melanogaster* proteins, respectively. Involvement of this gene product in cell cycle control has been confirmed by its ability to complement *S. pombe cdc25ts* mutant. Potential regulation of *cdc25+* mRNA in culture cells and animals is being characterized.

D 519 CELL CYCLE REGULATION OF A CDC2-RELATED PROTEIN KINASE, Vincent J. Kidd, Lisa Pouncey, and Donald E. Adams, Departments of Cell Biology and Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294

We have recently isolated and characterized a new CDC2-related protein kinase, $p58^{GTA}$. This protein kinase is 434 amino acids in size and contains three domains: a 74 amino acid "regulatory" domain, a 299 amino acid $p34^{cdc2}$-related domain, and a 61 amino acid carboxyl terminal domain. The protein is conserved in all organisms examined thus far, including human, murine, bovine, chinese hamster, and sea urchin. The $p58^{GTA}$ kinase is expressed ubiquitously and it is localized to both the cytoplasm and nucleus. Nuclear staining is punctate, and appears to be confined to the nucleolus. Like $p34^{cdc2}$, $p58^{GTA}$ appears to form a multimeric protein complex that is dynamically altered during the cell cycle. Overexpression of the $p58^{GTA}$ kinase in eukaryotic cells leads to alterations in the cell cycle and ploidy; namely a prolonged late telophase and substantially increased micronuclei. Analysis of $p58^{GTA}$ RNA expression during the cell cycle in HeLa cells has shown that it is up-regulated during late S- and G_2-phase. $p58^{GTA}$ kinase activity peaks in late G_2- and M-phase in HeLa cells. Thus, expression of the $p58^{GTA}$ kinase appears to be coordinately regulated with the expression of $p34^{cdc2}$. Many of the regulatory motifs of the $p34^{cdc2}$ kinase protein have been absolutely conserved in the $p58^{GTA}$ kinase. In addition, the $p58^{GTA}$ kinase is capable of autophosphorylation, and displays differential kinase activity towards specific substrates when it is dephosphorylated. Further study is now underway to determine the *in vivo* substrates of this kinase, as well as the mechanism(s) of its regulation during the cell cycle.

D 520 EXPRESSION OF THE MURINE HOMOLOGUE OF THE CELL CYCLE CONTROL PROTEIN $p34^{cdc2}$ IN T LYMPHOCYTES. Young H. Kim, Jacques J. Proust, Meredith J. Buchholz, Francis J. Chrest and Albert A. Nordin, Clinical Immunology Section, Gerontology Research Center, National Institute on Aging, NIH, 4940 Eastern Avenue, Baltimore, MD 21224

The mammalian homologue of the cdc2 gene of the fission yeast *S. pombe* encodes $p34^{cdc2}$ protein kinase that regulates the cell cycle of a wide variety of cell types. In the present studies, we investigated the expression of the cdc2 gene as well as the activity of the protein kinase during the activation and proliferation of murine T lymphocytes. Resting T lymphocytes prepared from spleens contained no detectable $p34^{cdc2}$ protein, histone kinase activity or specific mRNA of the cdc2 gene. Activation of the T cells by immobilized anti CD3 resulted in a rapid expression of specific mRNA and at G_1 phase of the cell cycle $p34^{cdc2}$ protein was detectable. However, there was no casein or histone kinase activity detectable at this phase of the cell cycle. As the cells progressed through cycle, the amount of specific mRNA increased, $p34^{dcdc2}$ was rapidly detectable and histone kinase activity was first detected at premetaphase. These results show that the expression of the murine homologue of the cdc2 gene is linked to activation through the T cell receptor and suggest that the kinase activity of the $p34^{cdc2}$ protein regulates the cell cycle of T lymphocytes in a manner similar to that shown for several other cell types.

D 521 THE STRUCTURE, EXPRESSION AND FUNCTION OF A SCHWANNOMA DERIVED GROWTH FACTOR, Hideo Kimura, Wolfgang H. Fischer and David Schubert, The Salk Institute for biological Studies, P.O. Box 85800, San Diego, CA 92186-5800

During the development of the vertebrate nervous system, neurons and glial cells require growth factors that regulate their division and survival. Specific proteins are thought to be released by target cells or the cells themselves in autocrine manner. To identify new growth factors for the nervous system, serum free growth conditioned media from a large number of clonal cell lines derived from the rat nervous system were screened for the presence of mitogens for CNS glial cells. One cell line which secretes a potent glial mitogen was recently established in our laboratory from a sciatic nerve derived Schwannoma. Schwann cells secrete an autocrine mitogen into their culture medium and human Schwannoma extracts have mitogenic activity on glial cells in culture. Neither mitogen has been purified. We purified and characterized a mitogenic molecule, designated Schwannoma derived growth factor (SDGF), from the growth conditioned medium of this Schwann cell line. This protein belongs to the epidermal growth factor (EGF) family and is an autocrine growth factor as well as a mitogen for astrocytes, Schwann cells, and fibroblasts.

D 522 EFFECT OF TGF-BETA ON THE PROLIFERATION OF B CELL LINES AND ON THE EBV INDUCED TRANSFORMATION OF B CELLS, Klein E, Altiok A and Maria Teresa Bejarano,.
Department of Tumor Biology, Karolinska Institutet, S-104 01 Stockholm, SWEDEN.

We tested the effect of transforming growth factor (TGF)-beta 1 and -beta 2 on the proliferation of human B cell lines. The panel was selected to give information whether 1) their origin 2) their phenotype 3) their Epstein-Barr virus (EBV) carrier state, influence their responsiveness. The growth of lymphoblastoid cell lines (LCL) was not inhibited by TGF-beta 1, while EBV carrying Burkitt lymphoma (BL) lines, Daudi, Jijoye, Rael but not Raji were inhibited. Three EBV negative BL lines and the majority of their sublines converted in vitro to the EBV carrier state. The cell lines tested expressed TGF-beta receptors and TGF-beta 1 transcripts in the in vitro EBV transformation system. The proliferation of B cells was inhibited by TGF-beta, their sensitivity decreased however after 3 days. The results suggest that the phenotype related to the activation state of the B cells influences TGF-beta sensitivity.

D 523 A MAMMALIAN PROTEIN KINASE WITH POTENTIAL FOR SERINE/THREONINE AND TYROSINE PHOSPHORYLATION IS RELATED TO CELL CYCLE REGULATORS. Kenneth Letwin, Yaacov Ben-David, Lisa Tannock, Alan Bernstein and Tony Pawson, Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, Canada M5G 1X5.

In a screen of mouse erythroleukemia cDNA expression libraries with antiphosphotyrosine antibodies, a strategy designed to isolate protein-tyrosine kinase coding sequences, we identified several cDNAs encoding proteins identical or very similar to known protein-tyrosine kinases. However, two frequently isolated clones, designated *clk* and *nek*, encode proteins which are most closely related to protein kinases involved in regulating progression through the cell cycle, and contain motifs generally considered diagnostic of protein-serine/threonine kinases. The *clk* gene product contains a C-terminal *cdc2*-like kinase domain, most similar to the *FUS3* catalytic domain. A bacterially exporessed *clk* protein becomes efficiently phosphorylated on tyrosine as well as serine and threonine in *in vitro* assays of kinase activity. Further evidence indicates that both these activities are intrinsic to the *clk* catalytic domain. These results suggest the existence of a novel class of protein kinases, with an unusual substrate specificity, which may be involved in cell cycle control.

D 524 CHARACTERIZATION OF HUMAN IL-5 RECEPTORS ON MYELOID CELLS, Angel F. Lopez, Susan E. Beltrame and Mathew A. Vadas, Division of Human Immunology, Institute of Medical and Veterinary Science, Adelaide South Australia, and Andrew Lewis, Linda S. Park, Immunex Corporation, Seattle, WA 98101.

Human IL-5 receptors were detected in peripheral blood eosinophils but not in neutrophils or monocytes. A single class of IL-5 receptors were found which were small in numbers (<1000 receptors/cell) and of high affinity (KD = 16-66 pM). Examination of a panel of myeloid cell lines revealed that HL-60 but not other cell lines, including B cell lines, expressed IL-5 receptors.

Specificity studies showed that mouse IL-5 fully inhibited the binding of human ^{125}I-IL-5 to eosinophils. Among human cytokines the two other eosinophil hemopoietic growth factors GM-CSF and IL-3 partially inhibited ^{125}I-IL-5 binding. Other cytokines including TNF-α, IL-1ß, LIF, IL-4 and IL-6 did not compete up to a concentration of 10^{-7}M. In reciprocal experiments IL-5 inhibited the binding of ^{125}I-IL-3 and ^{125}I-GM-CSF to eosinophils from only some individuals, perhaps reflecting IL-5 receptor occupancy by IL-5 *in vivo*.

The close association of IL-5 receptors with IL-3 and GM-CSF receptors on eosinophils may represent a unifying mechanism that could help explain the common biological effects of these factors on human eosinophil function.

D 526 IGF-II PARTIALLY REVERSES SURAMIN-INDUCED GROWTH INHIBITION OF RHABDOMYOSARCOMA CELLS, Caterina P. Minniti, Lee J. Helman, Department of Molecular Genetics, Pediatric Branch, NCI, Bethesda, MD, 20892.

Recently we have reported that IGF-II functions as an autocrine growth factor for Rhabdomyosarcoma cells. Suramin has been shown to neutralize the biologic effects of a several growth factors *in vitro*, including IGF-I. Therefore we studied the effect of suramin on the growth of the human rhabdomyosarcoma cell line RD. When increasing concentrations of suramin were added to exponentially growing RD cells in serum free medium we observed a dose dependent decrease of cell number. The IC50 was 125ug/ml which is in the range (150-300 ug/ml) of the reported clinically achievable dose of suramin. To determine whether suramin was affecting the rate of RD cell growth by interfering with the cellular binding of IGF-II, we added a large excess of exogenous recombinant IGF-II to RD cells in the presence of suramin. Under these experimental conditions we observed a 50% reversal of the suramin induced inhibition of cell growth. Since IGF-II exerts its biologic effects on RD cells by binding to the type-I receptor, we performed a radioreceptor assay using 125IGF-II and increasing concentration of suramin. This experiment demonstrated that suramin inhibited the specific binding of IGF-II to the cell surface in a dose dependent fashion.

D 527 TGF-beta GROWTH INHIBITION OF CULTURED BOVINE ANTERIOR PITUITARY-DERIVED CELLS IN CULTURE IS ACCOMPANIED BY DECREASED TGFα AND EGF RECEPTOR mRNA LEVELS, Susan G. Mueller and Jeffrey E. Kudlow, Department of Clinical Biochemistry, University of Toronto and Department of Medicine, University of Alabama at Birmingham.

TGF-beta is a multi-functional regulator of cell growth and differentiation. We report here that TGF-beta decreases the proliferation rate of non-transformed bovine anterior pituitary-derived cells grown in culture. We have previously demonstrated that these cells express both TGFα and its receptor (the EGF receptor) and that expression can be stimulated by phorbol ester (TPA) and EGF. Prolonged TGF-beta treatment (4 days) of the pituitary cells decreased cellular proliferation. This decreased growth rate was accompanied by a decrease in the TGFα mRNA level. The effect of TGF-beta on TGFα mRNA down-regulation was dependent upon cell density, the most pronounced effect observed when TGF-beta treatment was started on sparse cells. EGF receptor mRNA was similarly decreased by TGF-beta treatment. The reduction in the levels of TGFα and EGF receptor mRNA by TGF-beta was dose dependent, a maximal effect observed at a TGF-beta concentration of 1 ng/ml. These changes in TGFα and EGF receptor mRNA were relatively specific in that the hexosaminidase A or actin mRNA levels remained constant. Assuming that TGFα and its receptor play a positive role in the growth of the pituitary cells in culture, these experiments suggest that TGF-beta may inhibit the proliferation of these cells by acting as a negative regulator of these genes. These results also reinforce the concept that the effects of TGF-beta are cell specific, since in other cell types, TGF-beta has been shown to increase EGF-receptor expression.

D 528 **MOLECULAR CLONING AND EXPRESSION OF AN INTERLEUKIN-6 SIGNAL TRANSDUCER, GP130.**
M. Murakami, M. Hibi, M. Saito, M. Narazaki, T. Hirano, T. Taga, and T. Kishimoto, Institute for Molecular and Cellular Biology, Osaka University, 1-3, Yamada-oka, Suita, Osaka 565, Japan.
Among the members of cytokine receptor family, IL-6R has a relatively short cytoplasmic region, which was experimentaly proved unnecessary in IL-6 signaling. We have further found that IL-6 signal is transduced through a membrane glycoprotein, gp130, which associates with IL-6-R in the presence of IL-6. This association takes place extracelluarly, since soluble IL-6R lacking the transmembrane and cytoplasmic regions could associate with gp130 in the presence of IL-6 and mediate the signal. The cDNA for gp130 has been cloned, showing that gp130 is an integral membrane protein with ~900 amino acids (~600 outside). It was revealed that the extracellular region comprises 6 units of a fibronectin typeIII module. And part of this region shows similarity with other cytokine receptors having conserved 4 cysteine residues and a typical WSXWS motif. The intracytoplasmic region includes consensus elements required for GTP-binding, common to *ras* proteins. cDNA-expressed gp130 demonstrated that this molecule has no IL-6 binding property. A 7kb gp130 mRNA is expressed in a variety of cells even in the absence of IL-6R. A Jurkat transfectant with IL-6R cDNA showed mainly low-affinity IL-6 binding sites. However, after co-transfection with gp130 cDNA, both high- and low-affinity IL-6Rs were observed, confirming that gp130 is involved in the formation of high-affinity IL-6 binding sites. Furthermore, IL-3 dependent BAF.B03 cells which lack both IL-6R and gp130, when transfected with gp130 cDNA, become responsive to an IL-6-soluble-IL-6R complex, confirming that gp130 is involved in signal transduction.

D 529 ISOLATION OF HUMAN SUPPRESSOR GENES OF A *S. CEREVISIAE sst2*
Satoshi Nomoto[1], Jun Tsuji[1], and Kunihiro Matsumoto[2]
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Mating pheromone leads to cell-cycle arrest in the G1 phase in addition to other various physiological responses in haploid cells of *Saccharomyces cerevisiae*. Mutants of *sst2* show supersensitive phenotype to mating pheromone and no growth recovery in the presense of mating pheromone. One approach for isolation of mammalian genes which function in growth recovery is selection of dosage suppressors of *sst2* mutants. Initially, we constructed Jurkat cDNA libraries in yeast vectors which express the foreign cDNA under the control of the galactose-inducible promoter. We transfected one of them into an *sst2* null mutant. We isolated transformants which grew on galactose-containing media in the presence of a low concentration of α-factor and whose phenotype was plasmid dependent. Two distinct suppressors showed suppression of the phenotype of the *sst2* mutant only in galactose-containing media. Further characterization of the transformants and their cDNAs is presently in progress.

D 530 INDUCTION OF IL-1 PRODUCTION BY LIGANDS BINDING TO THE SCAVENGER RECEPTOR IN HUMAN MONOCYTES AND THP-1 CELL LINE,
Tessa Palkama and Mikko Hurme, Department of Bacteriology and Immunology, University of Helsinki, Haartmanink. 3 00290 Helsinki, Finland
In this study we demonstrate that ligand binding to the scavenger receptor activates monocytes to produce interleukin 1 (IL-1). Polyinosinic acid (poly I) and fucoidan, both ligands known to bind to the scavenger receptor, induced IL-1α and IL-1β protein production in human monocytes. THP-1 cells, which normally do not express scavenger receptors, were virtually unresponsive to poly I and fucoidan. PMA, which upregulates scavenger receptor expression in THP-1 cells, rendered THP-1 cells sensitive to IL-1 production by fucoidan and poly I. Polycytidylic acid, a structurally related compound to poly I, which does not bind to the scavenger receptor, had only a weak IL-1 inducing effect on monocytes and PMA-primed THP-1 cells. Scavenger receptor induced IL-1 production was reduced by pertussis toxin suggesting a G-protein mediated signal transduction pathway in scavenger receptor induced IL-1 production. H7, a protein kinase C inhibitor was able to abolish the scavenger receptor induced IL-1 production, while HA 1004, a preferential protein kinase A inhibitor, had no effect in scavenger receptor stimulated IL-1 levels. Foam cell formation via lipid accumulation through the scavenger receptor in human monocyte/macrophages is one of the earliest events in atherogenesis. Due to the potentially deleterious effects of IL-1 on the vessel wall, IL-1 produced by ligand binding to the scavenger receptor in human monocytes may play a role in the pathogenesis of atherosclerosis.

D 531 ELEVATION OF MEMBRANE ASSOCIATE TYROSINE PHOSPHATASE ACTIVITY IN DENSITY DEPENDENT GROWTH ARRESTED FIBROBLASTS, Poay Hiang Tong and Catherine Pallen, Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 0511.

Protein tyrosine phosphatases (PTPases) have the potential ability to reverse the cellular activities and the effects of growth factor receptor and oncogene tyrosine kinases. Structurally, PTPases can be divided into 2 classes: receptor-like and non receptor-like PTPases. The finding that the extracellular domain of a receptor-like PTPase (LAR) is homologous to the neural cell adhesion molecules (N-CAM) suggests that the interaction of like PTPases on neighbouring cells may serve as a signal to alter the catalytic activity of the enzyme. (Streuli, M. et. al.(1990) EMBO J.,9, 2399-2407).

We investigated whether cellular PTPase activity was affected by cell density. PTPase activity in Swiss 3T3 fibroblasts did not increase proportionally to increasing cell density. However, there was a sharp increase in PTPase specific activity when the cells were contact-inhibited at saturation density. The PTPase specific activity was membrane associated and a consistent 7 to 9 fold elevation was observed in growth arrested confluent cells over cells in the growth phase. It is possible that the regulated elevation of PTPase activity is involved in certain conditions of growth arrest.

D 532 PROPERTIES OF RECOMBINANT HUMAN LYMPHOTOXIN (TNF-β) AND ITS SITE-DIRECTED MUTANTS, Alan G. Porter, Reiner Jänicke, Chong Seng Loh, Stephen Fong and Cynthia R. Goh, Institute of Molecular and Cell Biology, National University of Singapore, Kent Ridge Crescent, Singapore 0511, Republic of Singapore

We have purified recombinant lymphotoxin (tumour necrosis factor beta: TNF-β) from E.coli. TNF-β is cytotoxic towards some but not all HeLa cell sublines. The dramatic (>1000-fold) difference in sensitivity of two HeLa sublines to the cytotoxic effect of TNF-β is due neither to the difference in the affinity of TNF-β with their receptors, nor to variation in interleukin-6 expression induced by TNF-β. Using mouse L-929 cells, the cytotoxicity and receptor binding affinity of TNF-β has been compared with its site-directed mutants bearing single amino acid substitutions. Conservative substitutions at two particular amino acid positions abolish receptor binding and cytotoxic activity of TNF-β. It is noteworthy that these two amino acid positions align with exposed loop regions in the known three-dimensional structure of the related tumour necrosis factor alpha (TNF-α), where substitutions also abolish the cytotoxic activity of TNF-α. Overall, our results placed in the context of studies in other laboratories, suggest that there are at least four distinct peptide sequences in TNFs involved in receptor binding and triggering second messenger pathways.

D 533 EPIDERMAL GROWTH FACTOR-INDUCED GROWTH INHIBITION IN MDA-468 HUMAN BREAST CANCER CELLS IS CHARACTERIZED BY ALTERED CELL CYCLE TRANSIT AND GENE EXPRESSION, K.A. Nagendra Prasad and Jon G. Church, Terry Fox Cancer Research Labs, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3V6.

Epidermal growth factor (EGF) is known to inhibit cellular proliferation in MDA-468 human breast cancer cells. As an initial approach to understanding the cellular mechanisms involved in EGF-induced growth inhibition in MDA-468 cells, EGF effects on cell cycle progression were studied. Flow cytometric analysis of DNA content indicated that EGF-treated cells accumulate in G1 phase with a concomitant reduction of cell populations in S and G2/M phases of the cell cycle. This apparent G1 arrest was confirmed by treating the cells with vinblastine, which inhibits cell cycle transit in mitosis. Furthermore, EGF treatment caused a significant reduction in DNA synthesis, which dropped to 35-40% of that of control cells, as measured by the incorporation of [^{3}H]-thymidine. The removal of EGF from the growth-arrested cells, resulted in a return to normal DNA synthesis within 3 days. In an attempt to more precisely locate the EGF-induced growth inhibitory event in G1, mRNA transcripts from genes known to be expressed in a cell cycle-dependent manner were examined by Northern analysis. Histone 3.2, a gene expressed upon the entry of cells into S phase, was down-regulated in response to EGF. The induced expression of proliferating cell nuclear antigen (PCNA), which is a cofactor for DNA polymerise δ, and is known to occur in late G1 or at the G1/S boundary, remained unchanged in response to EGF. These results suggest that EGF-induced growth inhibition is characterised by a reversible block at the G1/S boundary of the cell cycle of MDA-468 human breast cancer cells.

D 534 PHOSPHORYLATION OF THE DNA BINDING DOMAIN OF NONHISTONE PROTEIN HMG-I BY cdc2 KINASE: REDUCTION OF BINDING AFFINITY, **Raymond Reeves[*,+], Thomas A. Langan[#] and Mark S. Nissen[*], [*]Dept. of Biochemistry/Biophysics and [+]Program in Genetics and Cell Biology, Washington State Univ., Pullman, WA 99164 and [#]Dept. of Pharmacology, Univ. Colorado School of Medicine, Denver, CO 80262.**

Mammalian HMG-I is a DNA-binding chromatin protein that has been demonstrated both in vitro and in vivo to be localized to the A·T-rich sequences of DNA. Recently a novel binding domain (BD) peptide, "the A·T-hook" motif, has been described that mediates specific interaction of HMG-I with the minor groove of DNA in vitro. Inspection of the A.T hook region of the binding domain showed that it matches the consensus sequence for phosphorylation by cdc2 kinase. Here we demonstrate that HMG-I is a substrate for phosphorylation by purified mammalian cdc2 kinase in vitro. The site of phosphorylation by this enzyme is a threonine residue at the N-terminal end of the principle BD region of the protein. Labelling of mitotically blocked mouse cells with (32-P)-phosphate demonstrates that this same threonine residue in HMG-I is also phosphorylated in vivo. Competition binding studies show that cdc2 phosphorylation of a synthetic BD peptide significantly weakens its interaction with A·T-rich DNA in vitro. These findings indicate that cdc2 phosphorylation may significantly alter the DNA binding properties of the HMG-I proteins. Since many cdc2 substrates are DNA binding proteins, these results further suggest that alteration of the DNA binding affinity of a variety of proteins is an important general component of the mechanism by which cdc2 kinase regulates cell cycle progression. Supported by NSF Grant DCB-8904408 (R.R.)

D 535 INACTIVATION OF THE RB GENE IN HUMAN NON-SMALL CELL LUNG CANCER

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The retinoblastoma susceptibility gene (RB) is a tumor suppressor gene which has been linked to the development of several human malignancies. Loss or inactivation of this gene appears to be central to the pathogenesis of retinoblastoma, and has also been identified in osteosarcomas, soft-tissue sarcomas, small cell lung cancer, and other malignancies. In cooperation with the Lung Cancer Study Group we have studied alterations in the structure and expression of the RB gene in non-small cell lung cancer (NSCLC). DNA and RNA were extracted from specimens of tumor and uninvolved surrounding lung from 209 cases of NSCLC and studied by Southern and Northern blot analyses, respectively. Twenty-two tumors (11%) failed to express normal RB transcripts, 20 of these failed to express any detectable RB mRNA, and two expressed mRNAs of abnormal size. Rb transcripts of normal size were found in the uninvolved lung tissue from all of these cases, indicating inactivation of the gene occurred in the course of tumor development. Southern analysis revealed complex internal partial deletions of the RB gene in the two tumors which expressed truncated transcripts. Immunohistochemical analysis of the RB gene product revealed abnormal staining in 54 of 157 (34%) specimens analyzed, including 13/67 adenocarcinomas, 24/63 epidermoid carcinomas, and 12/20 large cell carcinomas. Analysis of the clinical outcome of these patients for possible correlation to RB gene inactivation is currently underway. These results indicate that the RB gene is inactivated in a significant number of NSCLC.

D 536 CELL CYCLE PROGRESSION AND RETINOBLASTOMA PROTEIN EXPRESSION DURING INDUCED ERYTHROLEUKEMIA DIFFERENTIATION, Victoria M. Richon, Xavier Busquets, Richard A. Rifkind and Paul A. Marks, DeWitt-Wallace Research Laboratory, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

The retinoblastoma protein, pRB, has been implicated in the regulation of differentiation and the control of cell cycle progression. In actively dividing cells the pRB phosphorylation state is regulated in a phase-dependent manner. The predominant form in G_0 and G_1-phase cells is under-phosphorylated pRB; whereas, in the S and G_2 phases, most of the pRB molecules are phosphorylated. The regulation of pRB level and phosphorylation state was investigated during the induction of differentiation in murine erythroleukemia cells. There is a critical time in late G_1 or early S phase when inducer must be present in order to induce differentiation. This is followed by prolongation of the subsequent G_1 phase, then resumption of progression through the cell cycle for several generations until the final arrest in G_1/G_0. During hexamethylene bisacetamide (HMBA)-induced differentiation there is an increase in the underphosphorylated form of pRB that correlates with the prolongation of G_1. As the cells resume progression through the cell cycle, pRB is present in the phosphorylated form and increases approximately 3-fold, while the RB mRNA increases approximately 5-fold. Therefore, pRB may be involved in the control of cell cycle progression during HMBA-induced erythroleukemia differentiation.

D 537 *WILD TYPE* P53 PROTEIN SUPPRESSES IN VIVO GROWTH OF A P53 NON-PRODUCER TUMOR

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Recent experiments suggested that the p53 protein, which has been shown to act as a dominant oncogene, can also function as an anti-oncogene. This apparent dual activity was resolved when it was found that *mutant* p53 can enhance the malignant process, whereas *wild type* p53 may function as a negative growth regulator. The goal of our research is to elucidate the physiological function of the p53 and evaluate its association with malignancy. To that end, we persue our research in the following directions:

In an earlier comparative study of p53 producer and non-producer cell lines, we found that the *mutant* p53 protein may facilitate malignant transformation. In that study we showed that reconstitution of *mutant* p53 expression in the mouse Ab-MuLV transformed p53 non-producer cell line, L12, changed the cells from those that develop regressor tumors into those that are lethal for the host. Furthermore we showed that *mutant* p53 may cooperate with the *ras* oncogene in transforming rat primary embryonic rat fibroblasts. In our present experiments we are studing the activity of *wild type* p53. For that purpose we have introduced *wild type* p53 into L12 cells and found that reconstitution of expression of *wild type* p53 in these cells, while not affecting cell proliferation in vitro, reduced their capacity to develop tumors in vivo. This suggests that *wild type* p53 in these cells functions as a tumor suppressor gene. The advantage of this experimental system is that *wild type* p53 expression can be monitored in a cell environment that lacks endogenous p53 expression.

D 538 PROGRAMMED CELL DEATH IN TUMOR CELLS, TRIGGERED BY MONOCYTES AND CONTROLLED BY GROWTH FACTORS AND HORMONES

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It is demonstrated with several human tumor cell lines , that supernatants (SU) harvested from monocyte/tumor cell (MO/TC) cocultures in a serum free medium can mimic most of the effects of a direct MO-challenge. SU not only contain signals inducing TC-death but also signals inducing growth inhibition as well as growth stimulation. The response of a given TC to this complex cytokine milieu is critically dependent on TC-density and the presence of hormones and growth factors. It is shown, that TC-death can be induced by SU exclusively at or above TC-contact density, suggesting TC-interactions as an essential part of the program leading to TC-death. TNF, which is present in SU, does not participate in the induction of TC-death. In contrary, with some TC, for which a growth inhibitory effect of TNF can be demonstrated, removal of TNF by affinity chromatography leads to enhancement of SU-induced TC-lysis. This is in accordance with our earlier observation, that growth inhibitory signals suppress the susceptibility of TC to induction of cell death by MO. Likewise, it is demonstrated in the present work, that the withdrawal of growth accelerating factors (EGF) can suppress TC-susceptibility, if such withdrawal leads to growth inhibition. This further supports the concept, that the decision on whether a TC will die or not is dependent on its growth state (1): growth accelerating signals favour and growth inhibitory signals cause suppression of the lytic process. Independently of signals affecting proliferation, it is shown, that hydrocortisone (HC) generally leads to reduced TC lysis-susceptibility. This is shown with basically highly susceptible clones of human melanoma and bladder carcinoma cell lines, the response of which is shifted by HC to the behaviour of basically resistant sister clones. In an attempt to determine whether TC are actively engaged in or passively subjected to the processes leading to lysis the proteinbiosynthesisinhibitor cycloheximide was found to prevent SU-induced TC-death. In conjunction with the above data on control of TC-death by cell density, growth factors and hormones this result supports the assumption, that MO induced cell death is an example of programmed cell death triggered by cellular interactions.

1) van der Bosch, J. et al.: Exp.Cell Res. 187, 185-192, (1990).

D 539 THE ROLE OF TRANSFORMING GROWTH FACTOR BETA (TGFß) IN THE REGULATION OF GROWTH OF PRIMARY RAT TRACHEAL EPITHELIAL (RTE) CELLS. Joyce E. Rundhaug, Thomas Gray, Ronald W. Steigerwalt, and Paul Nettesheim, National Institute of Environmental Health Sciences, P.O. Box 12233, Research Triangle Park, NC 27709

It was previously shown that TGFß is produced by high density RTE cultures, suggesting that TGFß may be responsible for the arrest of proliferation and the induction of terminal differentiation in these cultures. We and others have shown that RTE cells are highly sensitive to growth inhibition by TGFß. However, this conclusion is based solely on studies of early, low density cultures using a colony forming assay to quantify cell proliferation. The purpose of the present studies was to examine the role of TGFß in the regulation of cell proliferation in early as well as late primary RTE cultures. We show that 1-5 day old cultures (early logarithmic growth phase) are most sensitive to growth inhibition by exogenous TGFß. However, TGFß responsiveness rapidly diminishes during late logarithmic and plateau phases of growth. TGFß inhibits proliferation (DNA synthesis) and also accelerates cell death. The possibility that the onset of TGFß secretion by the cultures and the decrease in TGFß responsiveness coincide is being investigated. While our data indicate that TGFß is an important autocrine growth regulator in RTE cultures, they do not support the notion that TGFß causes the plateauing of growth in late cultures.

D 540 CELL CYCLE ALTERATION AND SECOND MESSENGER SIGNALING INDUCED BY THE LYMPHOCYTE CHEMOATTRACTANT FACTOR (LCF), Thomas Ryan, William Cruikshank, Gilles Bolduc, Hardy Kornfeld, and David Center, Pulmonary Center, Boston University School Medicine, Boston, MA. 02118.

We have previously reported that a CD4-cell-specific chemoattractant lymphokine (LCF) generated from histamine stimulated CD8 cells induced surface expression of the interleukin 2 receptor, and that these effects were blocked by costimulation with anti-CD4 antibodies. In these studies we investigated the growth-factor potential of LCF and possible mechanisms for signal transduction. LCF was purified from stimulated human T cell cultures by rsCD4 affinity chromatography and used in these experiments at a concentration of 1nM. Using the metachromatic dye acridine orange, it was determined that LCF stimulation of human T cells for 24 hrs induced a 15% increase in the number of cells containing elevated RNA levels. While LCF alone was unable to stimulate the presence of S phase cells or cell proliferation, costimulation of LCF with 0.1ug/ml PHA resulted in a 50% increase in thymidine uptake as compared with PHA alone. All effects of LCF were completely blocked by costimulation with rsCD4 (5ug/ml). Using the high CD4 expressing cell line SUPT1, we measured an increase in membrane associated protein kinase C (PKC) stimulated by LCF. The rise peaked by 30s and was back to control levels by 2 min. A reciprocal fall and rise in cytosolic PKC was also observed. In addition, we have observed that 1 min stimulation by LCF (as well as by HIV-1 gp120) induces the phosphorylation of eight different proteins, with a predominant band at 20kDa. From these studies we conclude that LCF stimulation can alter the cell cycle of resting human lymphocytes and may achieve this change by the induction of PKC translocation and protein phosphorylation.

D 541 EVALUATION OF THE ROLE OF EXTRACELLULAR MATRIX PROTEINS, POLYUNSATURATED FATTY ACIDS AND C-MYC EXPRESSION IN THE INHIBITION OF THE SERUM-FREE GROWTH OF EPITHELIAL CELLS BY TGF-β1, Donald J. Sarubbi, Ramaswamy Narayanan, Nitin T. Telang and Michael J. Newman, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110 and Breast Cancer Research Laboratory, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, New York 10021

Novel or modified serum-free media were developed for the anchorage-dependent growth of nontransformed murine mammary epithelial cells (MMEC) and Balb/MK murine keratinocytes respectively. Growth rates for both cell lines were similar in serum-containg and serum-free media. The serum-free media were used to evaluate potential mechanisms of epithelial cell growth regulation by type 1 transforming growth factor β (TGF-β1). The growth of MMEC and Balb/MK cells was reversibly inhibited 40-65% in a time- and dose-dependent fashion by TGF-β1 under both serum-containing and serum-free conditions. Constitutive over-expression of a transfected c-myc oncogene in MMEC did not result in loss of sensitivity to growth inhibition by TGF-β1. In addition, Balb/MK and MMEC growth inhibition by TGF-β1 was not potentiated by polyunsaturated fatty acids or reversed by vitamin E. Exogenous type V collagen was able to mimic the inhibitory effects of TGF-β1 on the serum-free growth of Balb/MK and MMEC. In contrast, collagen types I and IV, fibronectin and laminin did not inhibit the growth of these cells. The type V collagen used was not contaminated with TGF-β1, and subsaturating, but not saturating concentrations of type V collagen and TGF-β1 were additive with respect to Balb/MK and MMEC growth inhibition. These results demonstrate that nontransformed epithelial cell growth inhibition by TGF-β1 is mediated by mechanisms distinct from those observed with certain carcinoma and melanoma cells. Our results also suggest the possible involvement of type V collagen in Balb/MK and MMEC growth inhibition by TGF-β1.

D 542 CELL-CYCLE SPECIFIC EFFECTS OF ERYTHROPOIETIN, Jerry L. Spivak, Mary Ann Isaacs, Steven J. Noga, Husham Mishu, Evelyn Connor and Angelo Valle, Hematology Division, Johns Hopkins University School of Medicine, Baltimore, MD 21205

Erythropoietin is an essential growth factor for erythroid progenitor cells but its role in cell cycle-specific events in its target cells is unknown. To examine this issue, we employed an erythropoietin-dependent mouse erythroleukemia cell line, HCD-57, derived from spleen cells inoculated with a molecularly cloned, replication-competent Friend virus isolate free of spleen focus-forming activity. Synchronized cell populations in early G_1 (93%), late G_1 (69%), S phase (54%) and G_2/M (77%) were obtained from HCD-57 cells in log phase growth by centrifugal elutriation. The cell cycle time for HCD-57 cells was approximately 12 hours and was not influenced by the presence or absence of erythropoietin. Synchronized cells from any cell cycle phase could complete the cell cycle in the absence of erythropoietin but cells from early G_1 arrested in G_1 in the absence of the hormone. There was constitutive expression of c-myb, c-myc, c-fos and c-raf mRNA in synchronized G_1 cells but only c-myc expression increased within 1 hour after exposure to erythropoietin. Taken together, the data indicate that erythropoietin is required by HCD-57 cells during G_1 for cell cycle progression and induces or stabilizes c-myc mRNA expression during this phase of the cell cycle.

D 543 ROLE OF THE RETINOBLASTOMA PROTEIN AND *CDC2Hs* GENE IN CELLULAR SENESCENCE,
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Human diploid fibroblasts (HDF) have a finite lifespan at the end of which they become G1-arrested in a senescent state. Young HDF also become G1-arrested in a quiescent state when they are crowded or deprived of mitogens. Heterokaryon studies suggest that senescent and quiescent HDF contain a common inhibitor of entry into S phase that can be overridden by cells containing DNA viral oncogenes such as SV40 T-antigen, adenovirus E1A or human papillomavirus E7. The retinoblastoma protein, a putative inhibitor of proliferation that is inactivated by binding to the aforementioned oncogenes, has the properties expected of this inhibitor. Both senescent and quiescent HDF contain the unphosphorylated form of the RB protein, but following serum stimulation, only the quiescent cells are able to phosphorylate RB and enter S phase. These data suggest that failure to phosphorylate RB may be an immediate cause of failure to enter S phase in serum-stimulated senescent HDF. The human homologue of the yeast *cdc2/cdc28* gene is a candidate for the kinase that is responsible for phosphorylation of RB. We find that serum stimulation induces expression of the *cdc2Hs* gene in quiescent HDF but not in senescent HDF. The *cdc2/cdc28* gene is a critical cell cycle regulatory gene that is necessary for both entry into S phase and mitosis in yeasts. If the homologous gene in human cells is likewise necessary for entry into S phase, lack of expression of this gene in senescent cells would be an important reason for their failure to enter S phase.

This work was supported by NIH grant AG00947.

D 544 GROWTH-FACTORS REGULATE mRNA LEVELS OF A KEY ENZYME IN PHOSPHOLIPID SYNTHESIS.
Teresa G. Tessner, Charles O. Rock, *Gabe B. Kalmar, *Rosemary B. Cornell, and Suzanne Jackowski. Biochem. Dept., St. Jude Children's Research Hospital, Memphis, TN 38105 and *Dept. Chem./Biochem., Simon Fraser University, Burnaby, British Columbia, V5A1S6.
Growth-factors stimulate the turnover of phosphatidylcholine (PtdCho) and the proliferative state induced by growth-factors creates a demand for a net increase in membrane phospholipid formation. In cultured eukaryotic cells, PtdCho is the predominant membrane phospholipid and the precursor to the two other major membrane phospholipids, phosphatidylethanolamine and sphingomyelin. We have investigated growth-factor regulation of PtdCho metabolism during the G1 stage of the cell cycle in the colony-stimulating factor-1 (CSF-1)-dependent murine macrophage cell-line BAC1.2F5. The transient removal of CSF-1 arrests the cells in the G1 stage of the cell cycle; following re-addition of CSF-1, the cultures synchronously progess through the cell cycle. Metabolic labelling studies demonstrated that CTP:phosphocholine cytidylyltransferase (CT) is the rate-controlling enzyme for PtdCho biosynthesis in BAC1.2F5 cells. Northern analysis revealed that the amount of CT mRNA increased 4-fold within 15 min of CSF-1 readdition and remained elevated for 2 h. The increase in CT mRNA levels correlated with the onset of CSF-1-stimulated [^{3}H]choline incorporation into PtdCho. Measurement of CT mRNA turnover in the presence and absence of CSF-1 indicated that message stabilization is an important factor in determining the levels of CT mRNA. These data point to growth-factor regulation of CT mRNA abundance as an important response to increased PtdCho turnover and the demand for new membrane phospholipid during the cell cycle.

D 545 THE PHOSPHORYLATION STATE OF THE RETINOBLASTOMA PROTEIN IN G_0/G_1 IS DEPENDENT ON GROWTH STATUS.
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The product of the Retinoblastoma gene (RB) is a nuclear phosphoprotein which is thought to regulate the proliferation of cells. Its phosphorylation state changes with passage through the cell cycle and it has been proposed that RB protein in its hypo-phosphorylated form prevents cells proliferating. We have investigated the phosphorylation state of the RB protein in an actively-dividing human B-lymphoblastoid cell line and after cell cycle arrest caused by α-Interferon (α-IFN). Cells in different phases of the cell cycle were purified by flow cytometry and the phosphorylation state of the RB protein in the purified cells was analysed by western blotting. Cells with 2N DNA content from an actively dividing population contain RB protein ~60% of which is in the highest phosphorylated form whereas only ~20% of RB protein is phosphorylated in 2N DNA cells after inhibition by α-IFN. Therefore the phosphorylation state of the RB protein in cells with 2N DNA content is dependent on whether the cells are actively cycling or whether they are growth-inhibited. Our data is compatible with one of the possible mechanisms of RB action which is that dephosphorylation of the RB protein in G_1 allows cells to enter a quiescent state. In addition, the finding that logarithmically growing cells with 2N DNA content contain considerable amounts of phosphorylated RB protein suggests that these cells may be primed to enter the next cell cycle. This study also sheds light on the molecular mechanisms which may mediate the cytostatic effects of α-IFN. Funded by the Cancer Research Campaign and the Kay Kendall Leukaemia Trust.

D 546 GROWTH INHIBITORY SIGNALS ANTAGONIZING TUMOR CELL DEATH DURING MONOCYTE/TUMOR CELL INTERACTION,

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In a recently established serum-free in vitro system (1) it is demonstrated, that the susceptibility of various human tumor cells (TC) to the induction of cell death by elutriated human monocytes (MO) is critically dependent on TC-density and growth state. As determined by flow cytofluorometric cell cycle analysis, TC forced out of the cell cycle into G0 are no longer susceptible to the induction of cell death. This suggests, that processes essential for the lytic pathway can not take place in G0 cells. In this context it is a very important notion that, TC are driven into G0 during interaction with MO. The rate of accumulation in G0 increases with increasing MO-dosage. This explains our earlier findings of a maximum of tumor cell lysis rate at rather low MO:TC ratios of around 1:2 and of suppression of lysis at higher MO-dosages (1). The dying TC-population consists essentially of G1-cells. The low growth inhibitory potency of this situation (low MO-dose) suggests, that TC are not arrested in G1 under these circumstances, but rather continue to perform preparations for G1/S-transit until they die. It is likely that these preparations are a neccessary prerequisite for or part of the processes leading to cell death. At sub-confluent density, TC loose their lysis-susceptibility suggesting interactions between TC themselves being essential for expression of the program leading to TC-death. Apparently, the decision on whether to die or to survive is made in the target cell on the basis of its ability to respond to MO-challenge by arresting its own cell cycle in G0. The potential significance of these findings for the supposed function of mononuclear phagocytes in tumor defense lies in the notion, that selective elimination of cells is based on their inappropriate response to growth inhibitory signals ,which is the most important functional difference between tumorigenic and normal cells. However, TC driven into G0 - thereby mimicking normal cell behaviour - can escape this elimination. Since signals involved in MO/TC-interactions contribute to the accumulation of TC in G0, this leads t o selection for the survival of TC capable of switching between transformed and untransformed phenotype. 1) van der Bosch,J. et al.: Exp.Cell Res. 187, 185-192, (1990).

D 547 INTERACTION BETWEEN NEGATIVE GROWTH SIGNALS MEDIATED BY SURFACE Ig AND TGF-β IN SEVERAL MURINE B CELL LYMPHOMAS,

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We have been investigating the mechanism whereby cross-linking surface immunoglobulin (sIg) on certain murine B cell lymphomas leads to their growth arrest in G_1. The lymphoma cell lines, CH31 and CH33, are growth inhibited by cross-linking sIg, whereas cross-linking sIg on CH12 was little effect on growth. The negative signal in these cells has been shown not to require the activation of PKC or elevation of $[Ca^{++}]_i$, events which are thought to be involved in positive signalling mediated by sIg, or to involve the G proteins modified by either cholera or pertussis toxin. Interestingly, we have observed a differential ability of these cells to be growth inhibited by TGF-β; the order of sensitivity to TGF-β is similar to the cell's sensitivity to anti-Ig (CH31>CH33>CH12). In addition there is a marked synergy between the growth inhibition observed with anti-Ig and that of TGF-β. In fact CH12, which is normally not sensitive to growth inhibition by anti-Ig, displays dose dependent growth inhibition in response to anti-Ig when treated with a suboptimal concentration of TGF-β That is, in the presence of TGF-β, CH12 displays a functional phenotype similar to CH33 and CH31. TGF-β message can be detected in each of these lymphomas (±α-Ig), however, active TGF-β can only be detected in supernatants derived from anti-Ig treated cells (18 hrs). Preliminary evidence suggests that the steady state message level of TGF-β does not dramatically increase in response to anti-Ig. Experiments are in progress to examine TGF-β receptor expression on these cells in response to anti-Ig. Taken together these data suggest several interesting possibilities concerning the role of TGF-β in the growth inhibition of these lymphomas. One possibility is that anti-Ig induced growth inhibition is due to the autocrine production and response to TGF-β. Another intriguing possibility is that the signalling pathways employed by negative signalling mediated by sIg or TGF-β are interrelated and act in concert to growth arrest these cells in G_1.
(Supported by NIH grant CA41363 and ACS grant IM-495)

D 548 CARBOXYL TERMINAL AND PSTAIR PEPTIDE ANTISERA DEFINE DISTINCT SPECIES OF p34 CDC2,

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In an attempt to understand the role of p34 cdc2 in cell cycle regulation, we have used antibodies that recognize different pools of cdc2 that are characterized by differential phosphorylation, association with distinct sets of proteins, differences in histone H1 kinase activity and subcellular distribution. The carboxyl terminal peptide antisera recognizes hypophosphorylated cdc2 in cytosol and hyperphosphorylated cdc2 in nuclei, while the PSTAIR peptide antisera only recognizes hyperphosphorylated cdc2 in both cytosolic and nuclear fractions. Hypophosphorylated cdc2 in cytosol is associated with proteins that are not found to be associated with hyperphosphorylated cdc2 from either cytosol or nuclei. Our results support the idea that histone H1 kinase activity is inversely related to cdc2 phosphorylation. Immunofluorescent staining with the two antisera demonstrate two very distinct patterns of localization throughout the cell cycle. Taken together, our studies suggest that the phosphorylation state of cdc2 plays an important role in its association with other proteins, regulation of its kinase activity, and subcellular localization.

D 549 DISTINCT SIGNALLING PATHWAYS FOR EGF-UROGASTRONE IN SMOOTH MUSCLE RESPONSE SYSTEMS.

S.-G. Yang, M. Saifeddine, S. Mokashi, M. Chuang, D.L. Severson and M.D. Hollenberg, Department of Pharmacology & Therapeutics, University of Calgary, Faculty of Medicine, Calgary, AB, Canada T2N 4N1

We have characterized two guinea pig gastric smooth muscle preparations (longitudinal, (LM) and circular (CM) muscle: JPET 248, 384) that respond to EGF by contraction and we have studied one cultured smooth muscle cell system (rat A-10 cells: J. Cell Physiol 139, 524) wherein EGF, synergistically with Vasopressin, triggers arachidonate (AA) release. In these three systems, we have used inhibitors either of tyrosine kinase activity (Tyrphostin (TP) and genistein (GS)) or of diacylglycerol lipase action (U57,908) to assess the signalling pathway(s) whereby EGF acts. Whereas TP and GS at appropriately low concentrations selectively blocked EGF-mediated contraction,the two inhibitors did not affect EGF-mediated AA release in A-10 cells. In the LM preparation, U57,908 completely blocked contraction, but was without effect on EGF action in the CM preparation or in the A-10 AA-release assay. Our data point to three distinct signal pathways for EGF in smooth muscle, two requiring tyrosine kinase activity where one (LM) but not the other (CM) requires concurrent diacylglycerol lipase action; and a third, wherein the synergistic action of EGF with Vasopressin appears not to require tyrosine kinase activity.

D 550 Induction of Inflammatory Cytokines in Human Monocytes by IL-7

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IL-7 was initially characterized as a growth factor for cells of the B and T lymphocyte lineages. However, we have shown that human monocytes are capable of responding to IL-7 by expressing a variety of inflammatory cytokines. These cytokines include IL-1α, IL-1β, IL-6, TNFα, MIP-1β and NAP-1/IL-8. Both the levels of cytokine induced and the kinetics of induction are comparable between IL-7 and other monocyte activators such as IL-1β and LPS. The induction of IL-6 and MIP-1β by IL-7 was found to be inhibited by IL-4. These data suggest a role for IL-7 in inflammatory immune responses. Also, a transient system is being established to study signal transduction through the IL-7 receptor as a means of analyzing the mechanism of IL-7 action in monocytes.

Nuclear Events

D 600 cAMP-RESPONSIVE ELEMENTS MODULATE BASAL AND INDUCED TRANSCRIPTION OF THE MOUSE ORNITHINE DECARBOXYLASE GENE, Mitchell Abrahamsen and David R. Morris, Department of Biochemistry, University of Washington, Seattle, WA 98195

Transcription of the ornithine decarboxylase gene (ODC) is rapidly induced by external stimuli which elevate cAMP levels or by overexpression of the catalytic subunit of protein kinase A (PKA). Analysis of the ODC promoter has identified 3 regions clustered around the start site of transcription, at positions -175 (CRE1), -48 (CRE2) and +95 (CRE3), that are identical to the core motif of the cAMP-responsive element (CRE) and are conserved within the mouse, rat and human genes. We have examined deletions and mutations of these areas to assess their importance for regulation of the ODC gene by the catalytic subunit of PKA. Removal of CRE1 had no affect on the inducibility of the ODC promoter by PKA. Deletion or mutation of CRE3, which is downstream of the transcription start site, resulted in a 2-fold decrease in the inducibility by PKA. DNAase I footprinting revealed that mutation of CRE3 resulted in the loss of a footprint at this site, which correlates with the loss of inducibility after mutation of CRE3.

Mutation of CRE2 resulted in the total loss of activity and inducibility of the ODC promoter. This loss of activity was independent of the presence of CRE3. DNAase I footprinting revealed the presence of an extended footprint covering both CRE2 and the TATA box of the ODC promoter. Mutation of CRE2 resulted in the loss of the footprint at this element as well as its extension over the TATA box. Mutation of the TATA box prevented the extended footprint without affecting the ability of proteins to interact at CRE2. Gel mobility shift analysis demonstrated that the ability of large protein complexes to assemble on the ODC promoter was dependent on an intact CRE2. These data are consistent with a model that CRE2 is critical for the ability of the ODC promoter to form an active transcription complex.

D 601 REGULATION OF GENE EXPRESSION BY THE CATALYTIC SUBUNIT OF PROTEIN PHOSPHATASE-2A, Arthur S. Alberts, Judy L. Meinkoth, Marc Mumby[+] and James R. Feramisco, Departments of Pharmacology and Medicine, University of California at San Diego, Cancer Center, La Jolla, CA 92093-0636; [+]Department of Pharmacology, University of Texas, Southwestern Medical Center at Dallas, Dallas, Texas 75235-9041

The phosphorylation state of a cell reflects the relative activities of protein kinases, frequently activated by growth factor/receptor recognition or second messengers, and the antagonistic phosphatases. The reversible modification of serine, threonine and tyrosine residues induce conformational changes that regulate the biological effects of numerous proteins. We have studied the effects of microinjection of serine-, threonine-specific protein phosphatase-2A (PP-2A) on the expression of β-galactosidase (β-gal) in a series of mammalian cell lines containing an integrated lac Z gene under the control of different enhancers, *i.e.*, serum responsive (SRE), phorbol ester responsive (TRE), and cAMP responsive elements (CRE) as well as endogenous c-*fos* expression by immunoflorescence in normal fibroblasts (Meinkoth, 1990). Injection of PP-2A catalytic subunit completely abolished serum induced expression of β-gal, driven by the c-*fos* serum response element (SRE). Catalytic subunit did not ablate TPA or 8-bromo-cAMP/isobutyrl-methyl xanthine induction of TRE or CRE responsive enhancers, respectively. We are currently examining the phosphorylation state of proteins which bind the dyad-symmetry element of the c-*fos* promoter utilizing a photoactivated, biotinylated oligonucleotide containing the SRE.

Meinkoth, J. Alberts, A.S. and Feramisco, J.R. 1990 Proto-oncogenes in cell development. Wiley, Chichester (Ciba Foundation Symposium 150) p 47-56.

D 602 POTENTIATION OF JUN TRANSACTIVATION BY RAS ONCOGENE PRODUCT IN RODENT FIBROBLASTS, Bernard Binétruy and Michael Karin, dpt of pharmacology, School of Medicine, UCSD,La Jolla 92093

Previous investigations indicate that: 1/ the expression of *ras* oncogene leads to activation of genes containing an AP1 site in their promotor ; 2/ cotransfer of *ras* and *c-jun* genes induces oncogenic transformation of primary Rat Embryo Fibroblasts (REF) ; 3/ there is a strict correlation between transforming and transactivating functions of JUN proteins in these cells. We have analysed, by transient expression assays in REF cells, the effect of an activated RAS oncoprotein on JUN-mediated transactivation of an AP1-reporter gene . We found that, in the absence of *ras*, this transactivation is dose-responsive, ranging from 5 to 20 fold induction with 0.5 to 10 µg of *c-jun* expression vector ; in the presence of *ras*, although the maximal level of transasctivation is identical, it is reached at an input of 0.5 µg instead of 10 µg of *c-jun*. In these experiments the classical cooperation for transactivation between FOS and JUN proteins was observed, but RAS had no effect on FOS-mediated transactivation. Therefore the target of RAS on the AP1-dependant transactivation mechanism is likely the JUN protein itself. This hypothesis is strengthened by several additional observations, most important the ability of a JUN-GHF1 chimeric protein which lack the DNA binding domain and leucine zipper of cJUN to activate expression of a GHF1-dependant reporter is highly stimulated by cotransfection with *ras*. This experiment also demonstrates that the target for Ras action is the transactivation domain of cJUN. A biochemical modification of cJun induced by expression of the RAS protein could explain the cooperation between the two genes seen during neoplastic transformation of REF cells.

D 603 MUSCARINIC (Hml) COUPLED TRANSCRIPTIONAL ACTIVATION THROUGH AN AP-1 PROMOTER ELEMENT. Steven J. Busch, Mary Richards, Richard Jones, and Raymond Leppik. Marion Merrell Dow Res. Inst. 67,009 Strasbourg, CEDEX, France.
Murine A9L cells stably transfected with the human M1 muscarinic acetylcholinergic receptor Hml genome, expressed receptors at 328 fmol/mg cell protein with affinities for the cholinergic agonist carbachol, of 18 µM with a Hill coefficient (nH) of 0.88. Agonist binding was tightly coupled to activation of phospholipase C (EC_{50} 8 µM, nH=1.13) based on the generation of inositol monophosphate in the presence of 5 mM lithium. Transient transfection assays were included in which a bacterial chloramphenicol acyl transferase (CAT) reporter gene was placed under the control of an inducible AP-1 promoter to examine the effect of agonist binding on AP-1 regulated gene expression. We found that transactivation through the AP-1 site was induced in a dose-dependent manner with an EC_{50} of 20 µM but not in A9L cells with no Hml. Induction by carbachol was blocked by the antagonist pirenzepine with an apparent pKi of 7.7 µM (nH=0.83) for both the PI response and for the transactivation response and 8.21 (nH=0.91) for receptor binding. The time course for agonist-inducible transactivation through the AP-1 promoter exhibits a biphasic profile with an initial rapid phase extending up to 10 min. of carbachol treatment and a subsequent slower induction phase which extends linearly to at least 6h. Carbachol treatment also induced expression of c-fos and c-jun. We suggest that Hml activation may contribute to triggering the ontological cascade by activation of protooncogenes including c-fos and c-jun and their transactivation of genes with AP-1-like elements in their promoters.

D 604 DOMAIN ANALYSIS OF THE INDUCTION OF THE MOUSE CATHEPSIN L PROMOTER BY cAMP, Michael Y. Chang and Bruce R. Troen, Department of Internal Medicine, Veteran's Administration Medical Center, University of Michigan, Ann Arbor, MI 48109
The major excreted protein (MEP) of mouse fibroblasts is the precursor to a lysosomal acid protease (Cathepsin L) induced by malignant transformation, tumor promoters (TPA), second messengers (cAMP), and growth factors. As previously reported, we have cloned a fully functional gene for MEP from NIH 3T3 cells and constructed a series of transcriptionally active MEP-CAT (chloramphenicol acetyl transferase) plasmids that contain various regulatory regions of the MEP gene. Transient transfections of NIH 3T3 cells with MEP-CAT plasmids, which contain only sequences upstream of the transcription initiation site, demonstrate that CAT expression is not stimulated by phorbol esters, but is induced by cAMP. As few as the first 110 base pairs upstream of the transcription initiation site are sufficient to confer induction of the MEP promoter by cAMP. Additional studies suggest that cAMP responsiveness is localized to the region between base pairs -61 and -110. Sequence analysis of this region reveals multiple putative nuclear factor binding sites, including 3 AP-2 transcriptional factor binding sites and 1 EGR-1 factor binding site. In addition, our results demonstrate that two separate and distinct domains mediate basal activity of the MEP promoter. One region is within the first 110 base pairs upstream of the transcription initiation site and the other is between base pairs -175 and -273. These MEP-CAT constructs will allow us to study the induction of the MEP promoter by PDGF (platelet derived growth factor). We are employing additional deletion mutations, site-specific mutagenesis, and gel mobility shift assays to delineate further the elements involved in the regulation of MEP gene transcription

D 605 NUCLEAR TRANSLOCATION OF PROLACTIN OCCURS DURING INTERLEUKIN 2 DRIVEN T CELL PROLIFERATION, C.V. Clevenger, A.L. Sillman, and M.B. Prystowsky, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104
Prolactin (PRL) is required in the interleukin 2 (IL-2) driven proliferation of T cells (C.V. Clevenger, et al; PNAS 87:6460). IL-2 stimulation of the resting cloned T cell line L2 induces the enhanced secretion of previously sequestered PRL, and a subsequent translocation of PRL into the nucleus. EM studies utilizing colloidal gold-linked PRL indicate a sequential transport of PRL in IL-2 stimulated cells from the cell surface into endosomes and multivesicular bodies, and finally into the cell nucleus. This translocation occurs in the first six hours of IL-2 stimulation and is correlated with progression across the G1 phase of the cell cycle. A seven-fold increase in PRL-receptor and a two-fold increase in PRL-receptor mRNA also occurs during IL-2 stimulation. Incubation of cloned T cells with either IL2 or PRL induces proliferating cell nuclear antigen and interferon regulatory factor-1 mRNA synthesis. Thus, the IL-2 regulation of PRL secretion, re-uptake, and nuclear translocation appears to be required for clonal T-cell expansion.

D 606 REGULATION OF THE EARLY GROWTH RESPONSE KROX 20 GENE, Janelle Cortner and Peggy J. Farnham, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706.
We have mapped the serum responsive transcription initiation site of the murine early growth response Krox 20 gene and have noted that mRNAs from this site can be induced in quiescent or proliferating cell cultures with either serum or cycloheximide. This induction of Krox 20 mRNA increases dramatically with increasing cell density and correlates with the percentage of Go cells in the population. However, fractionation of the stimulated cells into stage-specific populations using both centrifugal elutriation and fluorescent-activated cell sorting shows that Krox 20 mRNA is found throughout the cell cycle. Thus, although the induction potential of Krox 20 is correlated with increasing numbers of quiescent cells in the population, the mRNA is induced throughout the cell cycle. We have shown that the induction of Krox 20 in proliferating cultures has both a transcriptional and post-transcriptional component. We are currently examining the transcriptional regulation of the human Krox 20 gene (Egr-2) and have preliminary evidence suggesting that the Egr-2 promoter can be regulated by either Egr-2 protein or by the retinoblastoma (RB) protein. Experiments designed to identify the sequence elements required for this regulation are in progress.

D 607 IL-2 PRODUCTION BY mAb D7.5-INDUCED HuT-78 AND HUMAN PERIPHERAL BLOOD LYMPHOCYTES IS DEPENDENT ON TRANSCRIPTION, TRANSLATION, AND TYROSINE PHOSPHORYLATION. John A. D'Orazio and Joan Stein-Streilein, Departments of Microbiology/Immunology and Medicine, University of Miami School of Medicine, Miami, FL 33101
D7.5 is a monoclonal antibody (mAb) that is postulated to bind a physiologic "trigger molecule" found on the surface of NK and T lymphocytes. Incubation of resting human peripheral blood lymphocytes (HuPBL) with mAb D7.5 (100 µg/ml) resulted in the appearance of interleukin-2 (IL-2) within 2 hours in the culture supernatant. Cross-linking of mAb D7.5 was not required for the response. Neither phorbol ester, mitogen, calcium ionophore, accessory cells nor exogenous lymphokines was required for stimulation of the cells. HuT-78 cells (human $CD4^+$ T lymphocyte line) secreted IL-2 into the incubation supernatant when incubated with mAb D7.5. HuT-78 did not secrete IL-2 spontaneously into its tissue culture media, nor did it release IL-2 when incubated with an irrelevant monoclonal antibody of the same isotype, IgG2a. In both the HuT-78 and resting HuPBL systems, IL-2 bioactivity appeared in the incubation media within 20 minutes following initial exposure to mAb D7.5. Maximal levels of IL-2 were obtained within 45 minutes to one hour. MAb D7.5-induced production of IL-2 was dependent both on *de novo* transcription and translation since actinomycin D and emetine independently blocked the response in both HuT-78 and HuPBL systems. Tyrosine phosphorylation (e.g. of the zeta chain associated with CD3) has been shown to be involved in T cell activation. A tyrosine phosphorylation step was integral to the pathway of triggering induced by the mAb D7.5 since genistein (4'-5,7-trihydroxyisoflavone), a specific inhibitor of tyrosine kinases, completely abrogated the response. Together, these data support the postulate that the interaction of the D7.5 antigen with mAb D7.5 (or its physiologic ligand) may identify an integral step in the activation of T lymphocytes.

D 608 **CHARACTERIZATION OF THE MURINE *JUNB* PROMOTER**, Rolf P. de Groot, Marcel Karperien, Johan Auwerx and Wiebe Kruijer, Hubrecht Laboratory, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.
The product of the *junB* gene, a gene homologous to the proto-oncogene c-*jun*, is a component of transcription factor AP-1. *JunB* expression is modulated by a wide variety of extracellular stimuli, such as serum, growth factors, phorbol esters and activators of protein kinase A. In order to study the molecular basis of this complex regulation, we have cloned the mouse *junB* gene from a genomic testis library, and characterized the *junB* promoter. Here we show that the *junB* promoter is activated by serum, phorbol esters and cAMP, and is subject to regulation by Jun/AP-1. Sequences located between -91 and -44 are necessary for induction. These sequences are recognized by at least three different protein complexes. Using footprinting analysis we show that one of these complexes probably contains transcription factor CTF binding to a CAAT box, while the other two are previously undescribed protein complexes binding to a G-C rich region and an "inverted CRE-like" element. This latter element is at least partially responsible for transcriptional activation of *junB* in response to TPA and cAMP.

D 609 INDUCTION OF PRIMARY RESPONSE GENES IN SWISS 3T3 CELLS BY HEAVY-METALS, Daniel E. Epner[1] and Harvey R. Herschman[2], Division of Medical Genetics[1] and Department of Biological Chemistry[2], University of California, Los Angeles, Los Angeles, CA 90024

We previously cloned a set of primary response genes, which we called TIS (TPA-Inducible Sequence) genes, from a cDNA library prepared from Swiss 3T3 cells which were treated with tetradecanoyl phorbol acetate (TPA) and cycloheximide. TPA, polypeptide growth factors, and serum induce transcription from the TIS genes. We recently found that cadmium and zinc elevate TIS gene mRNA levels in Swiss 3T3 cells in a concentration-dependent fashion. The time-course of TIS gene mRNA accumulation after metal exposure parallels that of metallothionein mRNA accumulation under the same conditions, which is delayed in comparison to the accumulation of TIS gene mRNA after treatment with other agents. The induction of the TIS genes by metal probably occurs through transcriptional activation. Metal treatment does not stabilize TIS gene mRNA, and actinomycin blocks the metal-induced TIS gene mRNA accumulation.

TIS gene induction by metal may either represent a physiologic, compensatory response, like transcriptional activation of the metallothionein genes by metal, or a pathologic deregulation or derepression of the TIS genes during the process of cell death. Studies are underway to determine whether the degree and timing of TIS gene induction by metal correlate with the degree and timing of metal-induced cell death. Future studies should clarify the mechanisms by which metal treatment leads to induction of the TIS genes.

D 610 mIg MEDIATED Ets-1PHOSPHORYLATION IN MURINE B CELLS, Constance L. Fisher, Jacques Ghysdael, and John C. Cambier. National Jewish Center for Immunology and Respiratory Medicine, Dept. Pediatrics, 1400 Jackson St., Denver, CO 80206

Recent studies have demonstrated that the nuclear protein, Ets-1, which is preferentially expressed in lymphocytes, binds to the long terminal repeat of Moloney murine sarcoma virus and HTLV-1 and regulates gene expression. The association of Ets-1 with DNA has been shown to be altered when the protein is phosphorylated. Thus, Ets-1 may regulate gene expression in lymphocytes and this activity may be determined by its phosphorylation state. To address the possibility that Ets-1 activity may be altered by mIg-mediated signal transduction, we analyzed the effect of mIgM and mIgD ligation on the phosphorylation state of Ets-1. Monoclonal anti-IgM or anti-IgD antibody stimulation of normal mouse B cells led to increased phosphorylation of Ets-1 within 2 minutes. This response was absolutely dependent on calcium mobilization and could be induced by elevation of intra-cellular free calcium using the calcium ionophore, ionomycin. Calcium release from intracellular stores was sufficient to mediate the phosphorylation of Ets-1. Treatment of resting B cells with IL4, TGFβ-1, IFNγ, anti-class I, or anti-class II antibodies did not induce Ets-1 phosphorylation. In summary, calcium mobilization from intracellular stores following mIgM or mIgD ligation provides a necessary and sufficient signal for activation of Ets-1 phosphorylation. This phosphorylation event may act in the alteration of gene expression during B cell activation.

D 611 CHRONIC ETHANOL EXPOSURE CAUSES INCREASED TYROSINE HYDROXYLASE GENE EXPRESSION IN N1E-115 NEUROBLASTOMA CELLS, Greg G. Gayer*†, Adrienne Gordon*†¶ and Michael F. Miles*¶, *Ernest Gallo Clinic and Research Center, San Francisco, CA 94110; Department of †Pharmacology and ¶Neurology, University of California, San Francisco, 94143

Ethanol-induced alterations in catecholaminergic neurotransmission appear to be involved in the behavioral responses to acute and chronic ethanol consumption. Since tyrosine hydroxylase (TH) is the rate limiting enzyme for catecholamine biosynthesis and is regulated by second messenger systems known to be modulated by ethanol, we have conducted a critical study on ethanol induced changes in TH gene expression. Northern and western blot analysis showed that a 3-day treatment with 100mM ethanol increased TH-mRNA and protein by 97% and 54% respectively, in the neuroblastoma cell line N1E-115. N1E-115 cells were stably transfected with a plasmid containing 773 bp of the TH-promoter fused to a chloramphenicol acetyl transferase (CAT) reporter gene. Subclones containing the TH-promoter showed ethanol-induced increases in CAT activity, suggesting that ethanol modulates TH gene transcription. Simultaneous treatment of transfected cells with ethanol and agents that increase intracellular cAMP produced an additive increase in CAT activity. Ethanol effectively shifted the cAMP dose-response curve for TH promoter activity to the left by 3 orders of magnitude. These results show that chronic ethanol has a prominent effect on basal and cAMP-regulated TH expression. N1E-115 cells were also stably transfected with a plasmid containing a cAMP response element fused to the CAT reporter gene. In these clones ethanol did not stimulate CAT activity. Therefore, other second messenger systems not involving cAMP may also be involved in ethanol regulation of TH expression. Ethanol-induced changes in TH expression may be a critical aspect of central nervous system adaptation to chronic ethanol exposure.

D 612 THYROID HORMONE RECEPTORS REPRESS ESTROGEN RECEPTOR ACTIVATION OF A TRE, Gerhart Graupner, Xiao-kun Zhang, Maty Tzukerman, Ken Wills, Thomas Hermann and Magnus Pfahl, Cancer Center, La Jolla Cancer Research Foundation, La Jolla, CA 92037

The identification of hormone response elements in the promoter regions of hormonally regulated genes has revealed a striking similarity between the estrogen response element (ERE) and a palindromic thyroid hormone response element (TRE) derived from the growth hormone gene promoter. In addition, this TRE was described as a strong retinoic acid receptor response element (RARE) for all three receptor subtypes: RARα, β and γ. We show here that the estrogen receptor is an efficient inducer of the TRE. Induction from a TRE dimer compared to the TRE monomer demonstrates considerable cooperativity. Both thyroid hormone receptors, TR_2 and TRβ are capable to repress the estrogen receptor (ER) on this TRE. Wild-type ER and TRs bind the TRE in the absence and presence of their ligand; however, TRs form a more stable complex with the TRE than does ER, as measured by gel shift assay. Furthermore, TR does not inhibit ER binding by heterodimer formation. We discuss a general mechanism of transcriptional control from TREs by thyroid hormone receptors where the unliganded receptor prevents access from ER and other related receptors to the TRE, thereby enhancing the ligand specific response of the TRE.

D 613 SEQUENCE-SPECIFIC DNA BINDING OF THE PROTO-ONCOPROTEIN ETS-1, Barbara J. Graves, Julie A. Nye, and Cathy V. Gunther; Department of Cellular, Viral and Molecular Biology; University of Utah School of Medicine; Salt Lake City, Utah 84103

The *ets* proto-oncogene family encodes a group of sequence-related nuclear proteins. In a study of cellular proteins involved in the transcriptional regulation of murine retroviruses in T-lymphocytes, we recently discovered that a member of the *ets* family, *ets-1*, encodes a sequence-specific DNA binding protein. Furthermore, we proposed, based on functional data, that *ets-1* is a transcriptional activator of mammalian type C retroviruses (Gunther et al. 1990, Genes Dev.4: 667-679). The full length *ets-1* ORF has now been expressed in bacteria to generate a 54 kD protein product. The *ets-1* protein binds DNA in a sequence-specific manner as assayed by mobility shift assays and methylation interference. The DNA binding domain within the *ets-1* polypeptide has been mapped by deletion mutagenesis to lie within the carboxyl-terminal 14 kD. This region, termed the ETS-domain, contains precisely the highly conserved sequences present in all *ets*-related genes. The amino acid sequence in this region bears no resemblance to the sequence configuration of well-characterized DNA binding motifs, thus we propose that *ets-1* and *ets*-related genes constitute a new class of eukaryotic DNA-binding proteins. Mixing experiments with the 54 kD and 14 kD *ets-1* polypeptides have failed to provide evidence of heterodimer formation, thus we speculate that the *ets-1* protein binds DNA as a monomer. We also have investigated *ets-1* protein-DNA interactions by methylation and ethylation interference experiments. Evidence for major groove contacts on one face of the DNA helix by the *ets-1* ETS domain will be presented.

D 614 THE REGULATION OF IFN SYSTEM: THE ROLE OF TRANSCRIPTIONAL ACTIVATOR (IRF-1) AND REPRESSOR (IRF-2), Hisashi Harada, Susumu Itoh, Keith Willison, Jun Sakakibara, Masaaki Miyamoto, Takashi Fujita and Tadatsugu Taniguchi, Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan

Interferons (IFNs) are a heterogeneous family of cytokines, originally identified as antiviral proteins. On the other hand, IFNs play a critical role in cell growth and differentiation. In order to elucidate the control mechanism of the IFN system, we have identified and characterized two novel transcription factors, termed IRF-1 (Interferon Regulatory Factor-1) and IRF-2, both of which specifically recognize the regulatory cis-elements of IFN-α, IFN-β genes and a set of IFN-inducible genes. In this study, we show that expression of the IRF-1 cDNA in the IRF-negative EC (Embryonal Carcinoma) cells results in the efficient activation of endogenous IFN-α genes, as well as co-transfected IFN-α, IFN-β and MHC class I promoters. Furthermore, activation of the endogenous and transfected genes is strongly repressed by the co-expression of the IRF-2 cDNA. Recently, we have identified the IRF-responsive cis-element in IRF-2 gene promoter. Moreover, IFN-β, IRF-1 and IRF-2 promoters contain a NF-κB binding site (or NF-κB like factor), which might play an important role in the expression of a wide range of signal-responsive genes. These findings emphasize the dual function of the IRF-responsive cis-elements as positive and negative regulators, and suggest the operation of a network by these transcription factors in the IFN system.

D 615 RELATIONSHIP BETWEEN THE ACTIVATION OF PROTEIN KINASE C AND C-FOS/C-JUN ONCOGENE EXPRESSION DURING DIFFERENTIATION AND RETRODIFFERENTIATION OF HUMAN MONOBLASTOID U-937 CELLS

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Human U-937 leukemia cells differentiate along the monocytic lineage following 72 h exposures to 12-O-tetradecanoylphorbol-13-acetate (TPA). This induction of differentiation is associated with adherence and loss of proliferation, as well as expression of monocytic markers. In contrast, culture of TPA-induced U-937 cells in the absence of phorbol ester for 21-36 d is associated with a process of retrodifferentiation. The retrodifferentiated cells detached, regained proliferative capacity and cellular parameters associated with differentiation of the monocytic phenotype were reverted to those levels observed in uninduced control cells. Since treatment of U-937 cells with TPA resulted in a rapid translocation of protein kinase C (PKC) from the cytosol to cell membrane fractions increased levels of membrane-associated PKC-activity persisted until 24-28 d. However, longer periods of incubation were associated with a return to the distribution of PKC in control cells. Activation of PKC has been implicated in the regulation of certain immediate early response genes, and in the present studies TPA rapidly induced c-fos and c-jun gene expression. Nuclear run-on assays revealed that both, c-fos and c-jun were transcriptionally induced by TPA in the presence of cycloheximide suggesting a direct activation of these genes probably via a phosphorylation signal. Levels of c-fos and c-jun transcripts remained elevated during periods of PKC-activation and also returned to that in control cells by 24-28 d when the cells began to enter retrodifferentiation. Staurosporine and H7, non-specific inhibitors of PKC, blocked TPA-induced adherence and growth inhibition, as well as increased expression of c-fos and c-jun. Taken together these findings point to a close relationship between the rapid and long term translocation (activation) of PKC and a long term c-fos/c-jun gene expression suggesting a key role in the maintenance and reversion of the differentiated monocytic phenotype.

D 616 USE OF SSCP AND PCR TO DETECT p53 GENE MUTATIONS IN SMALL CELL LUNG CANCER Charles H. Hensel, Rui Hua Xiang, Alan Y. Sakaguchi, and Susan L. Naylor, Department of Cellular and Structural Biology, The University of Texas Health Science Center, San Antonio, TX 78284.

Recent studies have suggested that the p53 oncoprotein might function normally as a tumor suppressor. Mutations in highly conserved regions of the p53 gene have been observed in numerous types of tumors and tumor cell lines. We studied genomic DNA from twelve small cell lung cancer (SCLC) cell lines by conventional Southern blotting and observed no gross alterations in the p53 gene. However, RNA and protein analyses indicated that p53 expression was abnormal or absent in 5 of 13 cell lines. To detect in a more sensitive manner p53 gene mutations in SCLC we utilized the single strand conformation polymorphism (SSCP) technique of Orita et al. (Genomics 5: 874, 1989). Using PCR primers for the most highly conserved regions of the p53 gene, including exons 4-9, we have identified p53 mutations in 5 of 9 small cell lung cancer (SCLC) tumor DNA samples and in 1 SCLC cell line. None of the mutations seen in tumor DNA samples were present in normal DNA from the same patients, indicating that these mutations were somatic in origin. Of the six mutations observed, two were found in exon 7, three were found in either exon 8 or 9, and one was found in exon 5 or 6. Nucleotide sequencing of one of the exon 7 mutations and one of the exon 8-9 mutations indicated that each was a C to T transition. In SCLC-6 the mutation resulted in substitution of serine for proline at amino acid 278 and in SCLC-4 substitution of tryptophan for arginine at amino acid 248 was the result. Both of these are nonconservative amino acid substitutions and are in regions of the p53 gene where mutations have been observed in other tumors. An additional mutation affecting the splicing of p53 pre-mRNA was observed in SCLC cell line H209. This mutation results in the utilization of a cryptic splice site 48 nucleotides 5' to the normal splice site and the insertion of 16 amino acids into the encoded protein.

D 617 **Analysis of L-*myc* expression and mRNA processing in human fetal development and in leukemia cells.**

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The *myc* proto-oncogenes are expressed in fetal tissues, and may play inportant roles in normal embryonic development and differentiation. We analyzed L-*myc* mRNA expression and processing in developing human fetal tissues by RNAse protection, Northern and *in situ* hybridization. In the human fetus, L-*myc* was expressed in several tissues: skin, kidney, lung, muscle, brain, kidney and also in the hematopoietically active spleen and thymus. In the brain, L-*myc* mRNA levels in the post-mitotic intermediate and cortical layers did not differ from those found in the periventricular layers harboring the mitotically active neuroepithelial precursor cells. Thus, L-*myc* expression in normal development is uncoupled from cell division. The 3.6 and 3.8 kb mRNAs, resulting from alternative splicing of L-*myc* intron I, appear to be the prevailing L-*myc* mRNA forms in normal developing tissues.

The fetal expression patterns of proto-oncogenes are often recapitulated in malignant tumors. Since L-*myc* is expressed in fetal hematopoietic tissues, we studied its expressed also in leukemic cells and in hematopoietic bone marrow. By RNAse protection analyses L-*myc* mRNA was detected in 3/10 primary leukemias, as well as in some leukemic cell lines. The expression levels were relatively high, comparable to those in fetal brain and kidney. We conclude that (1) the tissue-specificity of L-*myc* expression is not as strict as previously reported, and (2) L-*myc* may have a role in the regulation of hematopoietic cell proliferation and/or differentiation.

D 618 MAPPING A PHORBOL ESTER RESPONSIVE PHOSPHORYLATION SITE ON C-JUN: IMPLICATIONS FOR V-JUN ONCOGENICITY, Warren K. Hoeffler, Arthur D. Levinson, Department of Cell Genetics, Genentech, Inc., South San Francisco, CA 94080

C-jun is a cellular transcription factor known to mediate the regulation of gene expression in response to phorbol esters. Its viral homologue, v-jun, bears close structual similarity except for the deletion of 27 amino acids. To assess the function of the deleted region, human 293 cells were transfected transiently with expression vectors encoding c-jun, v-jun, or delta c-jun (a deletion mutant of the 27 amino acid region) with or without v-fos. The deletion resulted in more efficient heterodimer formation of jun with the fos protein. Additionally, the following responses of the heterodimer to phorbol ester treatment were impaired: increased binding to the consensus binding site on DNA, subsequent increased transcription from a promoter containing these sites, and increased phosphorylation of jun. A phorbol ester inducible phosphorylation site on c-jun was mapped to serine 73, adjacent to the region deleted in v-jun. We propose that the 27 amino acid region modulates the response of c-jun to phorbol ester (and presumably other signals) by regulating phosphorylation of serine 73, and the deletion of this region, as occurs in v-jun, impairs its ability to respond to the signaling pathway.

D 619 CONSTITUTIVE EXPRESSION OF c-*myc* ABLATES INHIBITION OF CSF-1-DEPENDENT GROWTH BY INTERFERON-γ BUT NOT cAMP. Elizabeth Hyland, Charles O. Rock, John L. Cleveland and Suzanne Jackowski. Department of Biochemistry, St. Jude Children's Research Hospital, 332 N. Lauderdale, Memphis, TN 38104

Colony-stimulating factor 1 (CSF-1) is a hematopoietic cytokine required for the growth and survival of mononuclear phagocytes. We used the murine macrophage cell line BAC1.2F5 to investigate the inhibition of the mitogenic response to CSF-1. Like normal macrophages, prostaglandin E_2 (PGE_2) and interferon-γ (INF-γ) block BAC1.2F5 cell division. PGE_2 elevates cAMP levels and treatment of the BAC1.2F5 cells with cAMP analogs mimicks the inhibitory effects of PGE_2. Expression of the c-*myc* gene is stimulated by CSF-1 early in the G1 phase of the cell cycle and is thought to be required for cell proliferation. Exposure of BAC1.2F5 cells to PGE_2, Bt_2cAMP, or INF-γ attenuates the accumulation of c-*myc* RNA stimulated by CSF-1. To determine whether diminished c-*myc* mRNA expression by Bt_2cAMP or INF-γ is a consequence or the cause of growth inhibition, we constructed BAC1.2F5 cell lines in which c-*myc* expression was independent of CSF-1 using a Moloney murine leukemia virus-derived vector containing the complete c-*myc* coding sequence and the *neo* gene as a selectable marker. Stable cell lines obtained from transfection with the expression vector constitutively produced c-myc mRNA but still required CSF-1 for growth and survival. These cell lines were refractory to growth inhibition by INF-γ but remained sensitive to the growth inhibition by PGE_2 and cAMP analogs. These data show that the attenuation of c-*myc* expression is the critical event in the inhibition of CSF-1-dependent growth by INF-γ but that regulation of c-*myc* mRNA levels does not play a determinant role in growth inhibition by cAMP.

D 620 REGULATION OF FOS STABILITY AND PHOSPHORYLATION IN EARLY G_1 BY PLATELET-DERIVED GROWTH FACTOR, Jon A. Jackson and W. Jackson Pledger, Department of Cell Biology, Vanderbilt University, Nashville, TN 37232.

Balb/c-3T3 fibroblasts become competent to synthesize DNA in a manner which is both dose- and exposure time-dependent with regard to platelet-derived growth factor (PDGF). PDGF induction of early gene expression occurs within 30-60 minutes of stimulation, but PDGF is required for 3-4 hours. We examined early response gene products in order to determine any effects PDGF might have on activity or post-translational state. We report here data on Fos, the protein product of the *c-fos* proto-oncogene, which is rapidly synthesized in response to serum or PDGF.

Northern blot analysis and immunoprecipitation of nascent Fos protein showed that the time course of Fos translation parallels its transcription. Fos was post-translationally modified within 30 minutes of synthesis into a form(s) with decreased mobility in SDS polyacrylamide gels. In pulse chase experiments, we demonstrated that the continued presence of PDGF in the medium maintained significantly higher levels of the Fos protein compared to PDGF-stimulated cultures released into medium without PDGF. Increased amounts of the stabilized protein correlated with the presence of the higher molecular weight form(s) of the protein, as recognized by Fos antibodies, in whole cell lysates. The phosphorylation of Fos also required the continued presence of PDGF, and correlated with the presence of an upper band in Fos radioimmunoprecipitations. We hypothesize that PDGF-dependent competence induction of Balb/c/3T3 cells may be in part through the biochemical modification of proteins required for mitogenic signalling during early G_1. Our data indicate that PDGF both induces and regulates the stability of Fos during early G_1 in Balb/c/3T3 cells.

D 621 DEVELOPMENTALLY REGULATED INDUCTION OF PDGF A-CHAIN mRNA IN NORMAL HUMAN FIBROBLASTS
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We have previously found that stimulation of normal neonatal fibroblasts with PDGF or EGF leads to a transient induction of PDGF A-chain mRNA and the synthesis of PDGF-AA proteins. This could imply the existence of an autocrine feedback mechanism to amplify the mitogenic signal under certain conditions. We have now studied the PDGF-BB mediated induction of PDGF A-chain mRNA in cell cultures from young and old donors, to clarify if the levels of induction are correlated to the donor age and replicative potential of the cells. We found that the increase in amount of PDGF-A-chain transcripts in response to PDGF-BB, only occurs in cells of embryonic and neonatal origin. This finding suggests that the possible autocrine mechanism is regulated in a developmentally fashion. Data will also be presented regarding the PDGF binding properties of the cells.

D 622 EXPRESSION OF A BOVINE GROWTH HORMONE SIGNAL PEPTIDE-v-SKI FUSION GENE IN CULTURED MOUSE CELLS,
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We have generated mouse cell lines which produce large amounts of a viral transforming protein, v-ski. Two plasmids were constructed in which the mouse metallothionine I promoter was used to direct expression of the v-ski oncogene or a bovine growth hormone signal peptide-v-ski fusion gene (bGH-v-ski). Stable mouse cell lines containing the plasmids were produced and v-ski expression evaluated. Both v-ski and bGH-v-ski genes were expressed at high levels in transformed cells. The v-ski protein product accumulated in the nucleus while bGH-v-ski protein was found to accumulate in the cytoplasm but was not secreted by the transformed cells despite accurate cleavage of the bGH signal peptide. The apparent molecular mass of cytoplasmic v-ski is slightly larger than that of nuclear v-ski due to N-linked glycosylation of the cytoplasmic v-ski protein at two positions. Inhibition of glycosylation does not result in either secretion or nuclear localization of cytoplasmic v-ski. The v-ski gene and the bGH-v-ski sequences were introduced in a retroviral vector and assayed for their ability to transform chicken embryo cells. Both retroviral vectors were capable of transforming cells but bGH-v-ski did so with reduced efficiency.

D 623 THE PROTO-ONCOGENE PRODUCT cREL IS A COMPONENT OF THE NFkB-ENHANCER BINDING COMPLEX IN B CELLS.
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cRel is the cellular cognate of the highly leukemogenic oncovirus, reticuloendotheliosis strain T (REV-T). c-Rel mRNA is overexpressed in mature B cells and transformed B cell lines. We shall demonstrate that cRel is a component of the HIV-LTR NFkB-enhancer binding sequence (GGGGACTTTCC) in several B cell lines. Furthermore, cRel expression is able to transactivate promoters containing the NFkB-enhancer sequence, but not a mutant NFkB-sequence. cRel association with the enhancer sequence is not coordinately regulated with the remaining NFkB binding complexes although cRel does appear to be present in cells in an activatable form similar to NFkB activity. Binding of cRel to the NFkB enhancer can be induced either by dissociation of the protein complex with deoxycholate/NP-40 or by phosphorylation by cAMP-dependent protein kinase, PKA. The association of a 40 kD M_r phosphoprotein as a potential inhibitor, possibly IkB, will be presented. The mechanism by which the early response proto-oncogene product cRel can regulate the transcription of NFkB-enhancer containing promoter will also be discussed.

D 624 TGFß Stimulates the Expression of Several Immediate Early Serum Response Genes

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Transforming growth factor ß has pleiotrophic effects on cell growth and metabolism. We have previously reported on the induction of *jun* transcription factors in TGFß-treated cells (Pertovaara et al., Mol. Cell. Biol. 9, 1255, 1989). Here we describe TGFß-stimulation of several immediate early mRNAs such as N10 (a putative nuclear DNA-binding receptor) and *Krox* 24 (a zinc finger transcription factor) among 78 serum-inducible cDNA clones isolated from mouse fibroblasts. Interestingly, positive responses are detected both in fibroblasts, which are growth-stimulated by TGFß and in epithelial cells, which are growth-inhibited. Although the degrees of induction of the mRNAs are less with TGFß than by serum, the kinetics of induction is surprisingly similar. Downregulation of protein kinase C by TPA and cell transformation by the *neu* or *ras* oncogenes dramatically reduces the TGFß-inducibility of most of these mRNAs. TGFß-specific mechanisms thus stimulate the expression of a subset of genes including transcription factors typical for growth activation of normal resting cells. The significance of the immediate early gene response to TGFß will be discussed.

D 625 NUCLEAR BINDING FACTORS OF HRAS1 AND IGH MINISATELLITES ARE TRANSCRIPTIONAL REGULATORY PROTEINS

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Minisatellites (VTRs, VNTRs) are distributed throughout the genomes of vertebrates and are often closely associated with genes and gene clusters. We and others have shown that the VTR 1000-bp downstream from the HRAS1 gene possessed modest enhancer activity (5- to 10-fold) in a very restricted range of cell lines. To investigate the molecular basis for this enhancer activity, we first determined by gel retardation assays that the VTR bound a specific nuclear factor. This protein, by gel shift pattern, recognition sequence, tissue distribution and molecular weight, proved indistiguishable from the previously characterized transcriptional regulatory factor, H2TF1/TC-IIB/EBP-1. Since H2TF1 was present in the principal cell line in which the VTR demonstrated enhancer activity (EJ), as well as in cell lines in which this activity was absent (NIH3T3, HeLa), additional determinants of VTR enhancer function were sought. Because expression of HRAS1 mutations can alter transcription of certain genes, and because the cell line EJ contains an HRAS1 mutation, we tested a CAT construct containing an HRAS1 VTR in NIH3T3 cells bearing an integrated copy of the HRAS1 gene from EJ. The VTR was responsible for 6-fold enhancement over control levels, a result comparable to that obtained in EJ. We concluded that enhancer activation of the HRAS1 minisatellite was dependent upon the HRAS1 p21 signalling pathway, and that this activation may ultimately be mediated through H2TF1. We have also begun to investigate the possibility that minisatellites may generally play a role in the control of gene expression. To this end, we have also recently shown that the VTR 2 kb upstream of the J segments within the immunoglobulin heavy chain locus bound a protein identical to MLTF/USF, a cellular factor originally described for the adenovirus major late promoter and related to the family of proteins which bind the μE3 box of the IgH enhancer (roughly 8 kb downstream from the VTR).

D 626 THE IDENTIFICATION OF 41 NOVEL IMMEDIATE-EARLY GENES IN REGENERATING LIVER AND INSULIN STIMULATED H35 CELLS

Thomas M. Laz, Kenneth L. Mohn, Anna E. Melby, Jui-Chou Hsu and Rebecca Taub, Howard Hughes Medical Institute, Department of Human Genetics, University of Pennsylvania, School of Medicine, Philadelphia, PA 19104

Liver regeneration provides one of the few systems for analysis of mitogenesis in the fully developed, intact animal. Subtracted cDNA probes and differential screening of cDNA libraries were used to isolate immediate-early genes in regenerating rat liver and insulin stimulated rat H35 hepatoma cells. We have characterized 341 differentially-expressed clones resulting in the identification of 52 immediate-early genes. These genes have been partially sequenced and the mRNA expression pattern analyzed by Northern blots. 41 of these genes appear to be novel. 37 of the 52 immediate-early genes are expressed in BALB/c 3T3 cells but only 10 of these genes were identified in previous studies with the fibroblasts. We have found known immediate-early genes (jun B, egr-1 and c-fos), known genes that are also immediate-early genes in liver (PEPCK, gene33, IGFBP), novel genes with known motifs (leucine-zipper and steroid receptor transcription factors), and genes with no homology to the sequences in the computer database. 15 of the 52 genes identified in the liver system show tissue-specific expression. The immediate-early growth response may consist of more than 100 genes. The proliferative response and the cell-type specific regulation of a cell may be determined by the specific set of immediate-early genes.

One of the most highly expressed tissue specific immediate-early genes in regenerating liver is the rat homolog of the IGF binding protein (IGFBP-28). The regenerating liver produces IGF-I, but we did not detect any IGF-I receptor mRNA during the first 24 hours post hepatectomy. However, some IGFBPs may act to enhance the activity of IGF-I independently of the IGF-I receptors. In concert with IGF-1, this IGF binding protein could play a paracrine or autocrine role in regulating growth during liver regeneration.

D 627 Extinction of PDGF A- and B-chain mRNA expression in somatic cell hybrids. Per Leveen , Christer Betsholtz, Bengt Westermark
Department of Pathology , University of Uppsala, Sweden.
We have performed experiments with hybrid cell lines in order to compare the regulation of PDGF A- and B-chain gene expression in one tumor cell line and one normal cell line. The hybrid cells were obtained as fusion products between the human melanoma cell line WM115 which expresses high levels of both PDGF A- and B-chain mRNA and the hamster fibroblast cell line Wq3H which has no detectable expression of PDGF B mRNA and a barely detectable level of PDGF A mRNA. Northern blot analyses of ten hybrid clones showed that clones containing the human PDGF A- and B-chain genes expressed A- and B-chain mRNA at levels which were 19 % and 18 %, respectively, compared to those of WM115. The clones without the human PDGF A gene expressed the hamster variant of this gene at the same low level as in Wq3H while still no PDGF B expression was detected. Treatment of the hybrid clones with the translational inhibitor cycloheximide (CHX) caused a marked and transient reinduction of PDGF-B mRNA only in clones which has retained the human PDGF B gene. In contrast, a weak induction of PDGF A-mRNA by CHX was detected in Wq3H as well as in all hybrid clones. These results indicates that labile trans-acting factors derived from the Wq3H genome control the levels of the A and B chain mRNA in the hybrids either at the transcriptional or the post-transcriptional level. The observation that only the *human* PDGF B mRNA is inducible by CHX in the hybrids indicates that the hamster PDGF B gene is in an inactive state out of control by trans-acting factors.

D 628 INTERACTION OF NUCLEAR FACTORS WITH preB AND B LYMPHOID-SPECIFIC ENHANCER ELEMENTS IN THE IMMUNOGLOBULIN HEAVY CHAIN ENHANCER. Towia A. Libermann[1,2] and David Baltimore[1,3], [1]Whitehead Institute for Biomedical Research, Cambridge, MA 02142, USA, [2]Present Address: Department of Medicine, Beth Israel Hospital, Boston, MA 02115, USA and [3]Present Address: Rockefeller University, New York, NY 10021, USA.
Transcriptional regulation of immunoglobulin heavy chain (IgH) gene expression plays a central role in all stages of B cell development. B cell specificity is apparently regulated by both positively acting enhancer and negative acting silencer elements. We have recently characterized two novel lymphoid-specific enhancer elements, π and μB, in the IgH enhancer. We have demonstrated that the π element is only active at the preB cell stage and is shut off upon B cell maturation, whereas the μB element is active during all stages of B cell development. We now present data on purification and characterization of proteins interacting with the π and μB enhancer elements. One of the proteins specifically interacting with the μB site, NF-μB, is expressed during all stages of B cell development as well as in monocytic cells, but is apparently absent from non-hematopoietic cells. Mutations that interfere with activity of the μB element also eliminate binding of NF-μB. Gel mobility shift experiments with the π enhancer element revealed multiple specifically retarded, tissue-restricted DNA/protein complexes. One of them, NF-π, is apparently expressed only in preB, but not in mature B or non-B cells and binds only to the wildtype, but not the inactive mutant π element. Further structural and functional characterization of these proteins will be presented.

D 629 PURIFICATION AND FUNCTIONAL ANALYSIS OF IκB, Hsiou-Chi Liou and David Baltimore, Whitehead Institute, Cambridge, MA 02142
Nuclear factor κB (NF-κB) is a transcription factor which binds to a cis-acting motif in the Ig κ enhancer as well as some other inducible promoters/enhancers. In cells which do not express Ig κ gene, NF-κB is present in the cytosol in an inactive form by being complexed with an inhibitor protein, IκB. Physiological stimuli such as phobol ester and bacterial lipopolysaccharide are very effective in dissociation of NF-κB from IκB and subsequent translocation of NF-κB from cytosol to the nucleus. Thus, the NF-κB/IκB system serves as a second message in a signal transduction pathway which transduce activation signals from cell membrane to nucleus.
Initial effort has been directed to work out a purification scheme for IκB protein in an attempt to obtain some pure IκB protein for peptide sequencing and subsequently cloning of the gene. Cytosol fraction prepared from rabbit lung tissue was used as starting material. Anion exchange DEAE and gel filtration chromatographies were first applied to purify NF-κB/IκB complex as a unit. The NF-κB/IκB complex was then dissociated with deoxylcholate and the individual components were separated on another anion exchange column. The peak IκB fractions were further purified through gel filtration chromatograghy which resulted in a high degree of purification. Several resins with different properties are currently tested for further purification of IκB to near homogeneity. The final goal is to clone the IκB gene so that the physiological interaction between NF-κB and IκB and how the NF-κB/IκB complex responds to environmental stimuli can be addressed.

D 630 PROTEIN KINASE C AND INTRACELLULAR CALCIUM ACTIVATE DISTINCT SIGNALING PATHWAYS SYNERGISTICALLY TO INDUCE GENE EXPRESSION. Bruce Magun, Philippe Lenormand, David Pribnow, and Karin D. Rodland, Department of Cell Biology and Anatomy, Oregon Health Sciences University, Portland, OR 97201- 3098

The transcriptionally active RVL3-VL30 element contains a triple repeat of TGACTCC, a sequence nearly identical to the AP-1 binding site. However, 12-*O*-tetradecanoylphorbol-13-acetate (TPA) stimulation was unable to elicit CAT expression from a construct containing these AP-1 like sequences upstream of the thymidine kinase promoter present in pTES. Endothelin, which activates protein kinase C and elevates intracellular Ca^{2+} in Rat-1 cells, was effective in stimulating CAT expression from the VL30-pTES construct. We attempted to assess the relative importance of these second messenger systems, by stimulating each pathway separately with exogenous agonists. We determined that neither stimulation of pkC by the tumor promoter TPA nor elevation of intracellular Ca^{2+} by the tumor promoter thapsigargin was sufficient to stimulate CAT expression from the VL30-pTES vector. When combined, the two tumor promoters induced a synergistic increase in CAT expression. Our data indicate that elevation of intracellular Ca^{2+} by thapsigargin was not required for full activation of pkC by TPA. First, TPA was able to stimulate expression of other genes in Rat-1 cells, indicating full activation of pkC. Secondly, thapsigargin synergized effectively with epidermal growth factor to stimulate CAT activity from the VL30-pTES construct in cells depleted of pkC activity by chronic TPA treatment . The synergistic interaction between elevated Ca^{2+} and TPA was also observed in the expression of the endogenous *transin/stromelysin* gene, which contains a conventional AP1 site. Interactions between elevated intracellular Ca^{2+} and activated pkC may represent a general mechanism for the full activation of some pkC-responsive genes.

D 631 c-sis PRODUCT (PDGF, B CHAIN) MODULATES THE EXPRESSION OF AN INTERFERON-INDUCED GENE (202) IN MURINE FIBROBLASTS. S. Martinotti, E. Toniato, A. Colagrande, E. Alesse, C. Alleva, A. Gabriele, P. Lengyel*, L. Frati and A. Gulino, Dept. of Experimental Medicine, Univ. of L'Aquila and of Rome, Italy and *Yale Univ., Molecular Biochemistry and Biophysics Dept., New Haven, CT.

Platelet-derived growth factor (PDGF) enhances the expression of the interferon (IFN)-activatable (2'5')oligoadenylate synthetase gene. PDGF induction requires IFN, since its action is inhibited by antibodies against IFN. We extended a similar investigation to a newly discovered cluster of IFN activatable genes (C202 cluster). Treatment of G-0-arrested Balb/c fibroblasts with recombinant PDGFB increases the expression of 202 gene reaching a maximum after 3 hs. In contrast, a significantly delayed 202 gene activation, with respect to the PDGF action, was observed after IFN treatment, peaking after 9 hs of treatment. While cycloheximide was able to overcome the IFN-induced 202 gene expression, PDGF action on the gene was independent on ongoing protein synthesis. Furthermore, PDGF was still capable of modulating 202 gene expression even in the presence of antibodies against IFN. These data suggest that PDGF and IFN regulate 202 gene through separate mechanisms. In oredr to test this assumption, we have derived a series of mutants of the 5' regulatory region of 202 gene driving the expression of CAT gene, to identify putative regions able to respond to the PDGF-mediated activation.

D 632 INDUCTION OF κB-BINDING PROTEINS WHICH ARE DISTINCT FROM NF-κB AFTER PHYSIOLOGICAL ACTIVATION OF A MURINE ANTIGEN-SPECIFIC T CELL CLONE. P.G. McCaffrey, C. Jamieson*, R. Sen*, and A. Rao. Dana-Farber Cancer Institute, Boston, MA, and *Brandeis University, Waltham, MA.

The transcription factor NF-κB has been implicated in expression of several T cell activation genes such as interleukin-2 (IL-2) and the IL-2Rα subunit (IL-2Rα). We have shown that NF-κB is induced in a non-transformed antigen-specific T cell clone activated by antigen or by antibodies to CD3 or the T cell receptor. In addition, we detect a second major inducible κB-binding activity which is distinct from NF-κB. Binding competition and methylation interference analysis indicate that the second complex contacts a subset of the bases which are involved in NF-κB binding. Unlike NF-κB, induction of the second complex is dependent on new protein synthesis. Depletion of the cells of protein kinase C abrogates induction of NF-κB, but does not affect the second complex.

The role of these κB-binding factors in promoter activation was investigated by transient transfection of IL-2Rα promoter-CAT contructs into T cells followed by antigen stimulation. While deletion of the κB site abolishes induction of promoter activity in response to T cell receptor stimulation, more detailed mutational analysis of the κB site revealed that several residues known to be important for NF-κB binding are not required for promoter induction. These results show that factors other than NF-κB can bind to the κB sequence *in vitro*, and suggest that such factors may also play a role in activation of the IL-2Rα promoter via the κB site *in vivo*.

D 633 MUTATIONS IN THE RESPONSE TO IFN AND IN THE REGULATION OF IFN PRODUCTION, Roslyn McKendry, Joseph John, Sandra Pellegrini, Ian M. Kerr and George R. Stark, Imperial Cancer Research Fund, Lincoln's Inn Fields, London, WC2A 3PX, UK.
We have established a cell line, 2fTGH, in which expression of the bacterial gene guanine phosphoribosyltransferase is controlled by IFNα. 2fTGH cells can be used to select for cell lines mutated in the IFNα signalling pathway. Mutagenesis followed by selection in 6-thioguanine and IFNα yields mutants which have lost their response to IFNα. We have already reported one such mutant, 11,1, and here report the isolation of a second uninducible mutant, U2. Genes normally induced by IFNα in 2fTGH can no longer be induced in U2 and the response to IFNγ is partially defective. While most genes respond normally to IFNγ, the 9-27 gene does not. Gel retardation assays show that none of the three transcription factors normally induced in 2fTGH cells by IFNα (E and M) or IFNγ (G) are induced in U2. Instead, extracts of untreated U2 cells give rise to a novel band that is induced further by IFNγ but not by IFNα. Band-shift complementation assays reveal that untreated or IFNγ-treated U2 cells lack a functional Eγ subunit of the complex transcription factor E but that IFNα-treated U2 cells do contain a function activated Eα subunit.

2fTGH cells can also be used to select for cell lines that abnormally produce IFN. After mutagenesis and selection in hypoxanthine, aminopterin, thymidine (HAT) medium, mutant cell lines producing autocrine IFN can survive. We have selected three such lines: B3, J4 and A8. B3 produces low levels of IFNβ and J4 produces IFNα. Further work on the regulatory mutations in these cell lines will be discussed.

D 634 PROTEINS ACTIVATE PHORBOL ESTER-RESPONSIVE PROMOTERS IN SHORT TERM ASSAYS, Judy L. Meinkoth*, Jeffrey A. Frost*, David W. Rose*, Frank McCormick+ and James R. Feramisco*, Departments of Medicine and Biomedical Sciences, University of California at San Diego, La Jolla, CA 92093* and Cetus Corp., Emeryville, CA 94608+.
Mammalian cell lines containing a marker gene (E. coli lac Z) under the control of serum-responsive (SRE) or tumor promoter-responsive (TRE or AP-1 binding site) promoters were constructed (Meinkoth, 1990). These cell lines (SRE and TRE cell lines) were used to assess the role of ras proteins in signalling events that result in transcriptional changes. Wild type and activated c-N-ras proteins were purified from baculovirus-infected insect cells and activated c-H-ras protein from E. coli. All three ras proteins induced β-galactosidase when introduced into the cytoplasm of rat2 TRE cells by microinjection. β-galactosidase expression (determined by X-gal staining) in response to injected ras proteins was greater than that induced by the phorbol ester, TPA, in the TRE cell line. In contrast, none of the ras proteins analyzed induced β-galactosidase expression in either rat2 or Balb/c SRE cell lines although the injected cells exhibited ras-specific morphological changes. Serum treatment induced β-galactosidase expression to a high level in both SRE cell lines. These results functionally define AP-1 or TRE containing promoters as ras-responsive. These data also suggest that SRE containing minimal promoters are not ras-responsive.
Meinkoth, J. Alberts, A.S. and Feramisco, J.R. 1990 Proto-oncogenes in cell development. Wiley, Chichester (Ciba Foundation Symposium 150) p 47-56.

D 635 MULTIPLE FACTORS INTERACT WITH THE C-FOS SERUM RESPONSE ELEMENT. **Richard Metz and Edward Ziff. Department of Biochemistry and Kaplan Center, New York University Medical Center, New York, NY 10016.**
We have analyzed proteins in HeLa and PC12 cell extracts that interact with the human c-fos gene serum response element (SRE; nt -332 to -282), a DNA element which mediates c-fos response to growth factor generated signals. HeLa and PC12 cell extracts form three specific protein-DNA complexes with the SRE oligonucleotide which are resolved by gel electrophoresis. Each complex represents different protein factors interacting with the SRE oligonucleotide.
By screening a lambda GT11 cDNA library with the different oligonucleotides containing portions of the SRE sequence we isolated 2 cDNAs whose gene products interact with the SRE DNA element. One of these encodes a 38 Kda leucine zipper protein which binds to the SRE probe, and whose gene product undergoes a posttranslational modification when PC12 cells are treated with cAMP. In addition its localization, shown by immunofluorescence, changes from cytoplasmic to nuclear. A second cDNA encodes a factor that binds to the SRE probe and is highly homologous to the previously identified E12 transcription factor. Antibody to the bacterially expressed protein blocks binding to the SRE of a protein in fractionated nuclear extracts which is distinct from SRF. These data suggest that multiple SRE binding factors may regulate c-fos gene expression at the SRE. A model for such regulation will be presented.

D 636 REGULATION OF GENE TRANSCRIPTION BY INTERLEUKIN 1, Steven B. Mizel, Marcio Chedid, Barbara K. Yoza, and James Brooks, Department of Microbiology and Immunology, Wake Forest University Medical Center, Winston-Salem, NC 27103

Interleukin 1 (IL 1), a macrophage-derived protein, induces specific gene transcription in a broad spectrum of cell types. In some cell types, for example, the murine pre-B cell line, 70Z/3, and the human natural killer-like cell line, YT, cAMP may serve as a second messenger for IL 1 in the activation of the transcriptional factor, NF-κB. Using cytosolic extracts from unstimulated 70Z/3 and YT cells, we have demonstrated that protein kinase A (PKA), as well as PKC, can induce the in vitro activation of the inactive cytosolic form of NF-κB and induce its translocation into isolated nuclei. In T cells, however, IL 1 does not appear to induce significant cAMP production. However, transfection of a T cell line, EL4 6.1 C10, with an expression plasmid (pSBL-PKIs) containing a cDNA for a specific protein inhibitor of PKA, termed PKI, abrogated the stimulatory effect of IL 1 on NF-κB driven gene transcription. When EL4 cells were transfected with an expression plasmid containing the CAT gene under the control of AP-1 responsive element and stimulated with IL 1, CAT activity was not observed. However, if the transfected cells were stimulated with IL 1 and TPA, CAT gene transcription was markedly enhanced over that obtained with only TPA stimulation. Forskolin or IBMX also synergized with TPA in the induction of AP-1 mediated gene transcription. Co-transfection with pSBL-PKIs resulted in an inhibition of the IL 1 enhancement of TPA-induced CAT gene transcription. Thus although IL 1 does not induce significant cAMP production in T cells, PKA appears to play a critical role in the control of IL 1-mediated activation of NF-κB and AP-1 in these cells.

D 637 **Functional Specialization Within Families of Transcription Factors: Dominant Roles of JunB and FosB in Regulation of Interleukin-2 Expression**, David R. Morris[1], Margaret Allen[1], Adriana Radler-Pohl[2] and Michael Karin[2]
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The phorbol ester-responsive transcription factor, AP-1, is a complex mixture of homo- and heterodimers composed of the protein products of the *jun* and *fos* gene families. Although all these complexes bind to the same heptameric DNA sequence, there may be gene- and cell-specific differences in the transcriptional responses to the various dimers. Using specific antisense oligodeoxynucleotides, we examined the roles of various *jun* and *fos* gene products in the regulation of the IL2 gene, whose regulatory region contains two AP-1 binding sites. Conditions that activate IL2 expression in both normal T cells and in a thymoma cell line (EL4) strongly induce expression of *jun*B, but do not influence basal expression of c-*jun* or *jun*D. In the case of the *fos* family, c-*fos* and *fos*B were strongly induced, but *fra*-1 mRNA was undetectable in either cell type. Antisense oligonucleotides complementary to either *jun*B or *fos*B inhibited activation of the IL2 enhancer, while antisense *jun*D or c-*fos* oligonucleotides, which strongly inhibited expression from a construct containing three copies of a consensus AP-1 site, had no effect on expression of the IL2 construct. These data suggest that JunB/FosB heterodimers and/or JunB homodimers are the preferred activators in the particular context of the IL2 enhancer in T cells. An antisense c-*jun* oligonucleotide was somewhat inhibitory, but this gene is expressed at low levels and is not induced under conditions that elevate IL2 expression. The behavior of a variant of EL4 cells, which does not induce IL2 in response to phorbol esters, was consistent with a dominant role of *jun*B and *fos*B in regulating expression of this gene. Although treatment of this variant cell line with phorbol ester induced c-*fos* mRNA and activated AP-1 DNA binding activity, the cells are severely impaired in expression of both *jun*B and *fos*B. Thus, at least one defect in IL2 expression in the variant line results from an inability to induce expression of *jun*B and *fos*B in response to activation of protein kinase C.

D 638 LOSS OF NORMAL WILD-TYPE P53 EXPRESSION RESCUES ras INDUCED GROWTH ARREST OF REF52 CELLS, Michael Mowat, Sean E. Egan, Arnold H. Greenberg, and Geoffrey G. Hicks, Manitoba Institute of Cell Biology and the University of Manitoba, 100 Olivia St. Winnipeg, MB, Canada, R3E 0V9.

Over expression of an activated ras gene in the rat embryo fibroblast line REF52 results in growth arrest at either the G1/S or G_2/M boundaries of the cell cycle. Both the DNA tumor virus proteins, SV40 large T antigen and Adenovirus 5 Ela, are able to rescue ras induced lethality and cooperate with ras to fully transform REF52 cells. We will present evidence that the wild-type activity of the tumor suppressor gene p53 is involved in the negative growth regulation of this model system. p53 genes encoding either a p53val^{135} or p53pro^{193} mutation express a highly stable p53 protein with a conformation dependent loss of wild-type activity and the ability to eliminate any endogenous wild-type p53 activity in a dominant-negative manner. In co-transfection assays these mutant p53 genes are able to rescue REF52 cells from ras induced growth arrest resulting in established cell lines which express elevated levels of the ras oncoprotein and show morphological transformation. Transfection of REF52 cells with ras alone or a full length genomic wild-type p53-plus-ras results in growth arrest and lethality. Further, expression of exogenous wtp53 alone appears to induce a similar growth arrest and is strongly selected against. We propose that the selective event for p53 inactivation or loss during tumor progression may be to overcome a cell cycle restriction induced by oncogene overexpression (ras). These results suggest that a normal function of p53 may be to mediate negative growth regulation in response to ras or other proliferative inducing signals.

D 639 THE ROLE OF AN OCTAMER FACTOR BINDING SITE IN EXPRESSION OF THE INTERLEUKIN-2 PROMOTER, Edward A. O'Neill, Betsy Frantz, Eric C. Nordby, and Michael J. Tocci, Department of Molecular Immunology, Merck, Sharp, and Dohme Research Labs, Rahway, NJ 07065
Activation of Interleukin-2 (IL-2) gene transcription is thought to play a central role in the regulation of the cellular immune system. We have been analyzing cis-acting sequences found within the human IL-2 promoter in order to elucidate the role they play in the regulation of IL-2 transcription. We have determined that a sequence (TATGTAAAAC) within the NFIL-2A promoter element (Durand et al.,1988. Mol.Cell. Biol.: 8, 1715) is a functional octamer factor binding site: OTF-1/NFIII from HeLa cells (O'Neill et al., 1988 Science: 241, 1210) or an NFIL-2A binding protein from Jurkat cells specifically bind a DNA fragment containing the NFIL-2A element yielding an identical DNA-protein complex upon polyacrylamide-gel electrophoresis; the affinity of the Jurkat NFIL-2A binding protein for the NFIL2-A element was increased 2 fold when the wild-type sequence was mutated to a high affinity octamer site (TATGCAAATA) and expression of a reporter gene driven by a promoter containing this sequence was increased 5 fold; a single base-pair substitution known to change this sequence from a high to a low affinity octamer (TATGCAATTA) reduced both the affinity of the NFIL-2A protein for the sequence and gene expression from a mutant promoter by 10 fold.

D 640 CELLULAR GENE TRANSCRIPTION REGULATED BY TUMOR SUPPRESSOR GENE EXPRESSION, Russell D. Owen, Junichi Hosoi, Jeffry Montgomery, Roger Wiseman, and J. Carl Barrett, Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709
Loss of tumor suppressor gene function is essential to the multistep progression of Syrian hamster embryo (SHE) cells to neoplasia. Immortalized, non-tumorigenic cell lines have been established by carcinogen treatment of SHE cells; these cells can suppress tumorigenicity in cell hybrids with highly malignant cells. Passage and subcloning of immortalized SHE cells have been used to establish variant cell lines that lack tumor suppressor activity, but remain non-tumorigenic. By screening cDNA libraries made from suppressor-positive cells, three differentially expressed mRNAs were identified (type II collagen, type IX collagen, H19). Nuclear run-on analysis showed that transcription rates for type II collagen and H19 were higher in suppressor-positive cells than in suppressor-negative cells. Genomic sequence analysis revealed the presence of a 10 bp motif in both the H19 enhancer and the 5' flanking region of the rat alpha1 type II collagen gene. Transient transfection experiments have been used to test the biological activity of specific DNA sequences. Gel retention analysis demonstrated the presence of nuclear factors that interact with this DNA sequence. Further characterization of the DNA binding activity and its potential role in tumor suppressor gene action will be discussed.

D 641 CHARACTERIZATION OF PRDI-BFi: AN INDUCIBLE FACTOR THAT BINDS TO THE PRDI REGULATORY ELEMENT OF THE HUMAN INTERFERON-β GENE. Vito J. Palombella, Andrew D. Keller and Tom Maniatis. Dept. of Biochem. and Mol. Biology, Harvard Univ., Cambridge, MA. PRDI-BFi is a poly(I)-poly(C) plus cycloheximide (CHX) inducible binding activity that binds specifically to the positive regulatory domain (PRD)-I within the interferon (IFN)-β gene regulatory element (IRE). Recently we have shown that unlike MG63 cells, PRDI-BFi binding activity is induced in Hela cells by CHX alone. Photoaffinity cross-linking and renaturation experiments revealed that PRDI-BFi is a protein of approximately 24-29 kDa. In addition, similar experiments have shown that PRDI-BFc, a constitutive PRDI binding factor, has a molecular weight of approximately 50 kDa. To determine the relationship of PRDI-BFi to IFN regulatory factor (IRF)-1, which also binds specifically to PRDI, we examined the effect of polyclonal antibodies against IRF-1 on PRDI-BFi binding activity. These antibodies had no effect on the binding activity of partially purified PRDI-BFi, but did block the formation of an IRF-1:PRDI specific complex. This, along with the fact that PRDI-BFi is CHX inducible whereas IRF-1 binding activity is blocked by this treatment, indicates that IRF-1 and PRDI-BFi are two different PRDI binding factors. Since PRDI-BFi is induced by CHX, the binding activity of this factor must require a specific modification event. To determine the role, if any, of phosphorylation in the binding activity of PRDI-BFi, crude nuclear extracts containing this factor were treated with potato acid phosphatase. We found that phosphatase treatment inhibited the binding of PRDI-BFi to the IRE and that the inclusion of phosphatase inhibitors prevented this loss in binding activity. This observation suggests that phosphorylation may play an important role in the binding of PRDI-BFi to the IRE. We have thus far been unable to activate PRDI-BFi binding from uninduced cells with cAMP-dependent protein kinase or a partially purified fraction of the double-stranded RNA-dependent protein kinase. We are currently purifying PRDI-BFi and PRDI-BFc in sufficient amounts to obtain amino acid sequence information as a means of cloning cDNAs encoding both these factors.

D 642 INTERFERON-INDUCIBLE TRANSCRIPTION FACTORS: PURIFICATION AND ACTIVITY, John Parrington, Matthew J. Guille, George R. Stark and Ian M. Kerr, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX, UK.
Transcription of the 6-16 gene is activated transiently by treating cells with type I (α/β) interferons. This activation requires an interferon-stimulable response element (ISRE) within the 6-16 promoter. This element, (5' GGGAAAATGAAACT 3'), but not one including, for example, the mutation 5' GGGAAATGACACT 3', is capable of conferring interferon responsiveness on marker genes. Specific complexes are formed between this element and pre-existing cytoplasmic factors whose DNA-binding activity and nuclear translocation are activated rapidly by interferon. We describe a greater than ten thousand-fold purification of one such factor(s) by the use of conventional and DNA affinity chromatography. This material specifically activates an ISRE-containing promoter in a cell-free transcription system. The activity of a number of additional constitutive and interferon *inducible factors* is being similarly investigated.

D 643 **NUCLEAR LOCALIZATION OF C-FOS, BUT NOT V-FOS PROTEINS, IS CONTROLLED BY EXTRACELLULAR SIGNALS.** Marc PIECHACZYK ,Pierre ROUX, Jean-Marie BLANCHARD, Serge CARILLO and Philippe JEANTEUR URA CNRS 1191, Laboratoire de Biologie Moléculaire, U.S.T.L., Place Eugène Bataillon, 34095 MONTPELLIER Cedex 5

We recently showed that the transport of the protein product of the c-fos proto-oncogene from the cytoplasm, where it is synthetized, into the nucleus, where it operates as part of the AP1 transcription complex, is not spontaneous but depends on the continuous stimulation of cells by serum factors. A labile protein, the effect of which is reversed by cyclic AMP, is responsable for retention of c-fos protein within the cytoplasm of serum-starved fibroblasts. In contrast, v-fos proteins transduced by murine retroviruses FBJ and FBR, which remain nuclear in the absence of serum factors, evade the translocation control.

In conclusion, our observations point to the existence of a new regulation level controlling c-fos gene expression and raise the possibility of a new type of contribution to the tumorigenic potential of two viral oncogenes.

Ref. : P. ROUX, J.M. BLANCHARD, A. FERNANDEZ, N. LAMB, Ph. JEANTEUR and M. PIECHACZYK.
Nuclear localization of c-fos, but not v-fos proteins, is controlled by extracellular signals (1990) Cell in press.

D 644 **THE C-MYC BASIC REGION IS A METHYLATION-SENSITIVE SEQUENCE-SPECIFIC DNA BINDING MOTIF.**
George C. Prendergast and Edward B. Ziff. Howard Hughes Medical Institute, Department of Biochemistry, and Kaplan Cancer Center, New York University Medical Center, 550 First Ave./MSB 397, New York NY 10016.

The hypothesis that c-Myc is a sequence-specific DNA binding protein is long-standing but has yet to be corroborated. We previously noticed similarity between a basic region of c-Myc and the basic motifs of other helix-loop-helix (HLH) and zipper proteins. Because the basic motifs in HLH and zipper proteins are required for specific DNA binding, this suggested that the region in c-Myc might encode a sequence-specific DNA binding function. Dimerization of HLH and zipper proteins is a prerequisite for DNA binding through the basic motifs. Therefore, we constructed a chimeric protein termed E6 in which the c-Myc basic motif is efficiently assembled into dimers through linkage to the HLH dimerization interface of the enhancer binding factor E12. We find that E6 dimers specifically bind to an element (GGACACGTGACC) similar to a HLH protein recognition site ("E box") also recognized by the putative transcription factors TFE-3 and USF. Binding is dependent upon the integrity of the c-Myc basic motif but repressed by inclusion of the C- or N-terminal c-Myc sequences in the construct, suggesting complex regulation of c-Myc DNA-binding activity by terminal domains. E6 is more discriminating of core and flanking residues in site recognition than TFE-3 and USF. Furthermore, under similar conditions, methylation of the core CpG in the E box recognition site has little effect on USF and TFE-3 binding, but strikingly inhibits binding by the E6 chimera. These data provide the first evidence that c-Myc encodes a motif with a sequence-specific DNA-binding function, and they support the hypothesis that c-Myc encodes a sequence-specific DNA-binding protein. Experiments to test the possibility that the E6 DNA-binding specificity is relevant to that of native c-Myc *in vivo* will be presented.

D 645 GROWTH FACTOR-RESPONSIVE TRANSCRIPTION FACTOR, EGR-1, IS ACTIVATED BY V-SRC *via* A SERUM RESPONSE ELEMENT INDEPENDENT OF PROTEIN KINASE C. Sajjad A. Qureshi and David A. Foster, The Institute for Biomolecular Structure and Function and The Department of Biological Sciences, The Hunter College of The City University of New York, 695 Park Avenue, New York, NY 10021.

egr-1 is a "primary response" gene encoding a transcription factor responsive to growth factors and other mitogens. Elevating the PTK activity of the v-*src* gene product, v-Src, leads to the transcriptional activation of *egr*-1. Promotor analysis of the *egr*-1 gene demonstrated that v-Src-induced *egr*-1 is *via* a serum response element. Since the target sequence of the *egr*-1 gene product is known, secondary targets of v-Src-induced signaling may now identified. *egr*-1 has been characterized as a phorbol ester-inducible gene, suggesting a protein kinase C (PKC) mediated signaling pathway for induction of *egr*-1. However, depleting cells of PKC by prolonged phorbol ester treatment, had no effect on v-Src-induced *egr*-1 gene expression. In addition, v-Src- and phorbol ester-induced *egr*-1 gene expression can be distinguished pharmacologically using inhibitors of protein kinases. Since v-Src is also capable of activating PKC-mediated signaling pathways (Spangler *et al.*, 1989. PNAS 86:7017), these data demonstrate that v-Src activates both PKC-dependent and PKC-independent signaling pathways.

D 646 INDUCTION OF FOS AND JUN GENES BY HEMATOPOIETIC GROWTH FACTORS.
Robert L. Redner, Gail A. Osawa, Angel W. Lee, and Arthur W. Nienhuis.
Clinical Hematology Branch, NHLBI, NIH, Betehsda, Md. 20892

Jun (c-jun, junB and junD) and fos (c-fos, fosB, and fra) proteins form heterodimers, each with the potential of acting as a transcriptional activator. It has been hypothesized that each dimer may exhibit unique transacting properties. If so, one would predict that any given phenotypic change should be characterized by a conserved pattern of jun and fos expression. To test this hypothesis we have assessed jun and fos RNA expression after stimulation of factor dependent hematopoietic cells. The myelomonocytic cell lines 32D and FDCP1 quiesce in G_0/G_1 after Interleukin-3 deprivation, and upon stimulation re-enter the cell cycle. 32D responds to Il-3 with rapid induction of jun-B and c-fos, followed by jun-D and fra-1, but no induction of c-jun. FDCP1 shows a much different pattern, with induction of c-jun, jun-D, and fra-1. To investigate the response of a single cell line to different physiological stimuli we used a 32D subclone engineered to respond to Colony Stimulating Factor-1. This subclone showed identical induction of jun and fos after stimulation with either CSF-1 or Il-3. The conservation of response of a single cell line, but the disparate patterns demonstrated by different cells, suggest a fundamental difference in both the regulation and function of the fos/jun heterodimers in these cells.

D 647 THE ADENOVIRUS TYPE 5 E1A ONCOPROTEIN SUPPRESSES THE INTERFERON - MEDIATED SIGNAL TRANSDUCTION PATHWAY. Nancy Reich and Michael Gutch, Department of Pathology, SUNY at Stony Brook, Stony Brook, New York 11794.

Interferons (α/β) induce a number of physiological changes in cells resulting in antiviral and antiproliferative responses. Interferons elicit these responses by binding to cell surface receptors and subsequently transducing a signal to the nucleus that activates transcription of a specific subset of genes. We have found that the signal transduction pathway of interferon can be suppressed by the activity of the adenoviral type 5 E1A oncogene product. The promoters of the interferon-stimulated genes possess an interferon-stimulated response element (ISRE) that is both necessary and sufficient for transcriptional activation by interferon. Transcriptional repression by E1A appears to be mediated through the ISRE sequence since expression of a plasmid construct containing the ISRE cloned into a heterologous promoter (HSV TK gene) is inhibited by co-transfection with the E1A oncogene. The interferon signal transduction pathway is mediated by specific ISRE-DNA binding proteins that are induced by interferon. The induction of these factors was analyzed in a cell line that constitutively expresses the E1A oncogene products. Mobility gel shift assays demonstrate an inhibition in the appearance of these DNA binding factors in the E1A expressing cell line. Therefore E1A may function by inhibiting the activation or DNA binding capability of interferon-induced transcription factors.

D 648 **TRANSCRIPTIONAL AND POSTTRANSCRIPTIONAL REGULATION OF C-FOS EXPRESSION BY THE SERINE/THREONINE SPECIFIC PHOSPHOPROTEIN PHOSPHATASE INHIBITOR OKADAIC ACID**

Axel Schönthal & Jim Feramisco, UCSD Cancer Center, La Jolla, CA 92093, USA

Phosphorylation events are major regulatory mechanisms of signal transduction pathways that control cell growth and differentiation. We analyzed the potential contribution of serine/threonine specific protein phosphatases to the regulation of the c-fos gene, a protooncogene that is involved in the regulation of cell growth and differentiation. By use of okadaic acid, an inhibitor of protein phosphatases 1 and 2A, we present evidence that expression of the c-fos gene is regulated by serine/threonine specific protein phosphatases. This control is exerted on the transcriptional as well as the posttranscriptional level. The results suggest that dephosphorylation of regulatory phosphoproteins is an important mechanism for the down-regulation of c-fos promoter activity and the rapid degradation of c-fos mRNA. Examination of two protein kinase pathways that are known to regulate c-fos expression indicates that okadaic acid acts synergistically with protein kinase C, but not with protein kinase A. Since inhibition of serine/threonine specific phosphatases increases protooncogene expression, these experiments further strengthen the view that certain protein phosphatases may act as negative regulators of cell growth.

D 649 INDUCTION BY GROWTH HORMONE OF C-FOS AND C-JUN EXPRESSION IN A VARIETY OF CELL TYPES WITH DIFFERENT REQUIREMENTS FOR DIFFERENTIATION.

Jessica Schwartz, M-L. Tsai, V. Sumantran. Dept Physiol, U Mich Med Schl, Ann Arbor MI 48109.

Growth hormone (GH) induces the transcription of c-fos and c-jun in 3T3-F442A preadipocyte fibroblasts and is one of the major factors required for differentiation of these cells into adipocytes. To evaluate whether these proto-oncogenes, which are associated with differentiation, are responsive to GH only in cells in which GH promotes differentiation, we investigated whether GH also induced the expression of c-fos and c-jun in related cells with varying differentiation requirements. All cells were studied when confluent, hence not dividing, and were rendered quiescent for 24 hr. They were incubated with GH for 30 min before total RNA was prepared and subjected to Northern blot analysis. In the related but non-differentiating 3T3-C2 cell line, induction of c-fos and c-jun in response to GH was comparable to that in the 3T3-F442A preadipocytes. GH also induced c-fos expression in non-differentiating NIH 3T3 fibroblasts. Induction of both c-fos and c-jun by GH was clearly evident in the fully differentiated 3T3-F442A adipocytes, although it was somewhat attenuated compared to the preadipocytes. Since 3T3-L1 cells require insulin or insulin-like growth factor I (IGF-I), *rather than GH, to differentiate into adipocytes*, we also evaluated the effect of these hormones on c-fos expression in these cells. GH, insulin and calf serum all induced c-fos expression in the 3T3-L1 preadipocytes. As in the 3T3-F442A preadipocytes, insulin appeared less effective than GH in inducing c-fos. Furthermore, in a 3T3-F442A mutant cell line, GI-16, which differentiates with serum but does not require GH for differentiation, both GH and calf serum induced c-fos expression. Taken together, these studies indicate that GH induces c-fos and c-jun in related cell lines which do not differentiate or do not require GH for their differentiation. This suggests that GH may be necessary, but not sufficient for 3T3-F442A cell differentiation. Furthermore, induction of c-fos and c-jun by GH may be associated with events other than cell differentiation in these cells.

D 650 REGULATION OF EXPRESSION OF TISSUE INHIBITOR OF METALLOPROTEINASES BY GROWTH FACTORS,

Renu R. Sharma, Hélène Roucheleau and Dylan R. Edwards, Department of Pharmacology, University of Calgary, Calgary, Alberta, Canada T2N 4N1

Tissue inhibitor of Metalloproteinases (TIMP) is a 28kdalton glycoprotein whose expression is maximally-induced at the transcriptional level in fibroblastic cells within 2-3h following exposure to growth factors such as basic fibroblast growth factor (bFGF) or tumor promoters. Transforming growth factor-ß alone does not exert an effect on TIMP expression, but stronly synergises with other agents to superinduce TIMP, while reciprocally suppressing growth factor induced expression of the metalloproteinases, collagenase and stromelysin. Using transient transfection assays of constructs linked to CAT reporter plasmids we have identified cis-acting elements within the murine TIMP gene that are involved in responses to growth factors. Our studies have defined a region proximal to the TIMP promoter that contains an AP1-like site (TGAGTAA) and an adjacent 23bp dyad symmetry element that are important in serum-responsiveness. In addition we have mapped positively and negatively acting elements within a 2.5kbp upstream region that acts as an enhancer. Responsiveness of reporter constructs to TGFß requires the presence of both promoter-proximal and upstream enhancer sequences. Gel retardation experiments indicate that the AP1-like site close to the TIMP promoter does not act as a canonical TRE (TPA-response element). These results indicate that multiple, cooperating cis-acting elements are involved in the response of TIMP to growth factors. Supported by Alberta Heritage Foundation for Medical Research and Medical Research Council of Canada.

D 651 IDENTIFICATION AND CHARACTERIZATION OF GENES INVOLVED IN SIGNAL TRANSDUCTION IN THE NERVOUS SYSTEM. C. Shea, S. Kunes and H. Steller, Dept. of Brain and Cognitive Science, MIT, Cambridge, MA 02139.
In order to understand cell communication in the nervous system, we are studying the interactions which occur in the visual system of the fruitfly *Drosophila melanogaster*. Innervation by photoreceptor cells appears to provide a signal to the optic lobe precursor cells which directs them to undergo mitosis and differentiate. In the absence of innervation by imaginal photoreceptor neurons, flies will display only rudimentary optic lobes. Our objective is to identify and characterize genes whose expression in optic lobes of the brain is modulated by innervation by the photoreceptors of the eye. In order to identify this class of genes we have transformed flies with a P element carrying a *lac Z* reporter gene. We have isolated a line of transformants in which the *lac Z* gene is expressed in a discrete subset of optic lobe neurons only if photoreceptors innervate the optic lobe and induce differentiation. We are using this line to search for mutants in which the *lac Z* staining pattern is changed or abolished. We have isolated several classes of mutations, including those with absent or aberrant *lac Z* staining, enlarged brains and abnormal imaginal discs. These may represent mutations in genes involved in the signalling pathway connecting photoreceptors and the optic lobes of the brain.

D 652 TISSUE-SPECIFIC EXPRESSION OF THE GENE ENCODING THE INSULIN RECEPTOR-RELATED RECEPTOR, IRR. Peter Shier and Valerie M. Watt, Department of Physiology, University of Toronto, Toronto, Ontario, Canada, M5S 1A8. The structure of IRR is predicted to be as related to the insulin receptor (IR) and type I insulin-like growth factor receptor (IGF-I R) as they are to each other, and less so to the other members of the protein tyrosine kinase superfamily. This relatedness spans both the external, ligand binding portions of these transmembrane proteins, as well as the intracellular domains containing the protein tyrosine kinase activity. Whereas the IR is known to be expressed in a wide variety of tissues and cell lines, the distribution of IRR is far more restrictive. We have now isolated a portion of the rat IRR gene and used it to study the tissue-specific expression of IRR. Northern analysis has identified several tissues with two detectable transcripts, only one of which is large enough to encode the entire predicted primary sequence of IRR. Analysis of PCR amplified products has confirmed the distribution of IRR transcripts in these tissues. Isolation and analysis of a cDNA encoding IRR confirmed the predicted primary sequence of IRR which we had originally deduced from genomic DNA sequences. Analysis of the promoter regions of both the guinea pig and the rat IRR genes revealed elements typical of mammalian promoters. The presence of a putative TATA box is in contrast to the regulatory sequences found in the promoter of the IR gene and is in agreement with a non-housekeeping role of IRR *in vivo*. The evidence for expression of the IRR gene and the similarity of the primary structure of the protein encoded by it to those of IR and IGF-I R strongly suggest that IRR is a receptor for a ligand(s) of the insulin family. Isolation of the cDNA encoding IRR will thus facilitate determination of its function.

D 653 POTENTIAL ROLES OF GENES ALTERED ON CHROMOSOME 10 IN ASSOCIATION WITH HUMAN GLIOMAS. Peter A. Steck, Mark A. Pershouse, Azra Hadi, W.K. Alfred Yung, and T. Elton Stubblefield, Departments of Neuro-oncology and Molecular Biology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030.

The vast majority of high grade human gliomas (glioblastoma multiformes; GBMs) have been shown to exhibit a loss of heterozygosity for polymorphic alleles on chromosome 10. The biological significance of these non-random losses is unknown, although the modulation of tumor suppressor genes has been proposed. To address this hypothesis, we have inserted a normal chromosome 10 into cloned GBM cells which have no intact chromosome 10 (as assessed by karyotypic analyses) by microcell mediated chromosomal transfer. The hybrid GBM clones were selected on the basis of a functional adenosine kinase, an enyzme whose gene maps to chromosome 10 and that was made defective in the orginal GBM cells by treatment with tubercidin. The hybrid cells contain an intact chromosome 10 (by karyotypic and RFLP analyses), displays a flatter morphology, and have an increased number of cellular processes similar that seen for normal brain derived cells. The saturation density of the hybrids was decreased and the cells exhibited a cobblestone morphology at high density as compared to the parental GBM cells that showed extensive over- and under-lapping. The growth rate of the hybrid and parental cells was not significantly different. However, the hybrid cells exhibited, at least, a 50-fold decrease in their colony forming ability under archorage-independent conditions. Their ability to form tumors in animals is currently under investigation. These results suggest that the alterations in genes associated the chromosome 10 in gliomas may play a role in the cell's tumorigenic phenotype, rather than cellular proliferation.

D 654 SELECTION OF MID-G1 INDUCED AND REPRESSED cDNAs, Sean V. Tavtigian and Barbara J. Wold, Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125.

A significant number of cell cycle immediate early gene products are either known or suspected to be transcriptional regulatory proteins. One approach to understanding their role in G1 is to identify their regulatory targets. The targets of any specific immediate early gene product are expected to include a subset of those genes whose transcript prevalence is altered by mid-G1. A bank of such clones can be rescreened with cDNA probes prepared from growth arrested cells which inducibly overexpress individual immediate early gene products. Accordingly, we have used appropriately subtracted cDNA probes to differentially screen cDNA libraries constructed from both growth arrested and mid-G1 cells. Selected cDNAs were characterized for the timecourse of their expression following serum stimulation of growth arrested cells; we are currently screening these for the subset whose expression is regulated, either directly or indirectly, by c-myc protein.

D 655 THE ROLE OF FREE CYTOSOLIC CALCIUM (Ca_i) IN ACTIVATION OF SPECIFIC GENES BY PLATELET-DERIVED GROWTH FACTOR (PDGF), R.W. Tucker, D. Ferris, Johns Hopkins Oncology Center, 600 N. Wolfe Street, SB 210, Baltimore, MD 21205

Growth factors induce a cascade of gene activations, starting with the "immediate early genes" (e.g. c-fos, c-jun, jun-b, 77, and 268) that are induced within 30 minutes in the absence of protein synthesis. C-fos and c-jun proteins form a dimer that binds to specific sites within DNA and stimulates the increased expression of additional genes. In order to determine whether c-fos and c-jun are stimulated by separate and distinct intracellular pathways, digital image analysis of intracellular Fura 2 fluoresecnce was used to calculate Ca_i changes, and in-situ hybridization using ^{35}S-RNA probes was used to measure mRNA accumulation in single cells. One hr treatment of quiescent BALB/c 3T3 cells with PDGF (30 ng/ml) stimulated competence for DNA synthesis in 60% of cells, and increased levels of Ca_i and mRNA accumulation of all the immediate early genes in 100% of cells. Following chelation of intracellular calcium by Quin 2 or Bapta, PDGF still stimulated increased expression of c-jun and 268, but not of c-fos, 77, and jun-b. Chelation of heavy metals by TPEN did not inhibit activation of any immediate early genes. Quin-2 and Bapta may inhibit phospholipase C (PLC) activation by PDGF, thereby preventing increases in IP_3 (and Ca_i) and diacylglycerol. Thus, activation of c-fos may depend on PLC activation, while c-jun is controlled by other intracellular mediators. The subsequent cooperation between c-fos and c-jun proteins is a striking example of the synergy between intracellular second messengers induced by PDGF.

D 656 SEQUENCES MEDIATING TRANSCRIPTIONAL RESPONSE TO MESODERM INDUCING FACTORS. Peter D. Vize and Douglas A. Melton. Department of Biochemistry and Molecular Biology, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA.

The homeobox containing gene Mix.2 is expressed in a brief pulse during blastula and gastrula stages of *Xenopus* development. Mix.2 is 75% identical to Mix.1 at the amino acid level, and is expressed in a similar temporal and spatial pattern. Like Mix.1, the expression of Mix.2 is rapidly induced in animal cap cells by mesoderm inducing growth factors. In order to investigate the mechanisms of mesoderm induction, we have instigated a study of the pathway of Mix.2 transcriptional activation by mesoderm inducing agents.

Mix.2 genomic clones were isolated and sequences upstream of the AUG, including the cap site and TATA box, were fused to a CAT reporter gene. These plasmids are injected into one cell embryos, and cultured until development proceeds to the blastula stage. Animal cap cells, which do not normally express Mix.2, are cut from these embryos and cultured in the presence or absence of PIF(activin), a powerful mesoderm inducing molecule. CAT activity is present at high levels only in caps treated with PIF. This assay has been used to determine the PIF responsive region of the Mix.2 promoter, and the details of the sequences involved and the general mechanism of gene activation during mesoderm induction will be discussed.

D 657 TGF-ß TREATMENT OF HEP 3B CELLS DECREASES THE ACTIVITY OF A 31 kDa PROTEIN WHICH RECOGNIZES AN NF-1 BINDING SITE. Robert H. Whitson, Yuan Qian, and Keiichi Itakura, Dept. Molecular Biology, City of Hope, Duarte, California 91010.
Transcriptional activation of the α2(I) collagen promoter by TGF-ß requires the presence of an NF-1 binding site (NBS) (Rossi, *et al.*, *Cell* **52**:405 (1988)). Using "southwestern" blots and gel mobility shift assays, we have examined the effects of TGF-ß on proteins which recognize the NBS. Nuclear extracts were prepared from NIH 3T3 and human hepatoma (Hep 3B) cells which were treated with 200 pM TGF-ß for 24-48 hours. Extract proteins were resolved on polyacrylamide gels and transferred to nitrocellulose. The blots were then incubated with a concatamerized 32 base-pair DNA probe containing an NBS. Blots of control extracts from both cell types showed a strong signal at 31 kDa. On blots of extracts from hep 3B cells treated with TGF-ß for 24 and 48 hours the 31 kDa signal was reduced by 50 and 78%, respectively. TGF-ß did not affect the 31 kDa protein in 3T3 cells. The TGF-ß-sensitive 31 kDa protein appears to be specific for the NBS, since it was not detected on blots incubated with unrelated probes. Extracts of both cell types formed multiple complexes with the NBS probe in gel mobility shift assays, but no changes were seen in extracts from control *versus* TGF-ß treated cells.

D 658 CO-PURIFICATION OF FACTORS BINDING TO THE MAJOR LATE INITIATION SITE AND DOWNSTREAM SEQUENCE ELEMENTS OF THE SV40 LATE PROMOTER, Steven Wiley, Richard Kraus, Elizabeth Murray, Karla Loritz and Janet E. Mertz, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706

The SV40 late promoter contains three sequence elements necessary for transcription initiation at nt 325, the major cap site. One maps approximately 30 bp upstream, one spans the major late initiation site, and one maps approximately 30 bp downstream of the major initiation site. We have purified fractions from Hela cell nuclear extracts by affinity chromatography with a 17 bp oligonucleotide homologous to the sequences surrounding the major late initiation site. The resulting fraction contained an activity that protected this sequence from DNAse I cleavage in the absence of any upstream binding activity. Surprisingly, this extensively purified fraction also protected the SV40 major late downstream element from DNAse I cleavage. The purified fraction protected neither the initiation site nor the downstream sequence elements of the murine DHFR promoter. SDS-PAGE of the fraction showed three dominant proteins whose functions in sequence specific binding and transcription are currently under investigation. We conclude the following: i) The cellular factors that recognize the genetically significant initiation site and downstream elements of the SV40 major late promoter may interact by protein-protein association; ii) These combined activities may enhance stable binding to the promoter; and iii) Binding of these factors can occur in the absence of upstream binding activities.

D 659 **THE ROLE OF *fos* IN *v-src* INDUCED MITOGENESIS**, Anne W Wyke, Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD, U.K.
Mitogenesis induced by pp60$^{v\text{-}src}$ is not accompanied by detectable increases in transcription of the *fos* and *jun* genes in contrast to the response of the same cells to serum mitogens (M.J.Welham, J.A.Wyke, A.Lang and A.W.Wyke, 1990, Oncogene, 5, 161-169). However either stimulus results in induction of an AP-1 binding activity which is independent of *de novo* protein synthesis indicating a need for pre-existing Fos and Jun proteins in this response. Using anti-sera raised against Fos and Jun I have confirmed their participation in this complex and have also shown the need for protein phosphorylation for its formation. Does this *in vitro* increase in AP-1 binding have *in vivo* consequences? Mitogenesis induced by either serum or *v-src* results in imcreased levels of the AP-1 responsive rat stromelysin gene. Are these AP-1 changes required for *v-src* induced mitogenesis? The effects of controlled expression of *fos* sense and anti-sense RNAs in these cells are now under investigation.

D 660 CONSTITUTIVE DNA BINDING FACTOR(S) ESSENTIAL FOR INDUCTION OF THE GM-CSF GENE BY ANTIGEN STIMULATION IN JURKAT CELLS

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The expression of GM-CSF gene as well as other cytokines is activated by a combination of phorbol ester (PMA) and calcium ionophore (A23187) which mimics T cell activation in the human Tcell leukemia cell line, Jurkat. We have previously identified the upstream regulatory sequences which respond to PMA and A23187. One of these responsive sequences is located between -96 and -72 upstream of mouse GM-CSF gene (CLE2/GCbox)[1]. This region consists of two DNA binding motifs. One is the inducible protein (NF-GM2) binding site (GM2) and the other is the constitutive proteins binding site (GC box, CCCCCGCCCCCC)[2]. In the mobility shift assay using GC box sequence as a probe, we have identified three retarded bands (A1, A2, B) and the binding specificities of the A1, A2, and B bands are indistinguishable from each other. The requirement for both sites for the stimulation of the GM-CSF gene by PMA and A23187 is absolute in in vivo transfection assay and the constitutive site is absolutely required in in vitro transcription assay. The A1 protein is identical to the Sp1 protein since the formation of the A1 band is abolished by the addition of antibody against Sp1 protein (obtained from Dr. Tjian's) into the binding mixture but not those of A2 and B bands. Furthermore, the A1 band is reconstituted by the addition of the purified Sp1 protein. The involvement of the Sp1 protein and/or other proteins which recognize the GC box sequence in in vitro transcription is under investigaton.

1)Miyatake,S. et.al. Mol. Cell. Biol. 8:5581-5587, 1988.
2)Sugimoto,K. et.al. Int. Immunol. 2:787-794,1990.

Late Abstracts

MITOGEN-INDUCED REPETITIVE FREE Ca^{2+} TRANSIENTS IN SINGLE NIH-3T3 FIBROBLASTS,

Greg J. Barritt, Anthony J. Polverino and Bernard P. Hughes, Department of Medical Biochemistry, School of Medicine, Flinders University of South Australia, G.P.O. Box 2100, Adelaide, South Australia, 5001

Changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}{}_i]$) induced by mitogens in individual NIH-3T3 fibroblasts loaded with fura-2 were investigated. At 3 mM extracellular Ca^{2+} ($Ca^{2+}{}_o$), bombesin induced three types of response: a single transient increase in $[Ca^{2+}]_i$ (29% of cells tested), a sustained increase (14%) and repetitive transients (49%). Eight percent of the cells gave no response. Fetal calf serum (FCS) induced similar heterogeneous types of responses although the repetitive transients were more irregular in both amplitude and period. An increase in the concentration of bombesin from 1 to 25 nM increased the percentage of cells which gave repetitive Ca^{2+} transients, decreased the lag before commencement of the transients and decreased the period of the transients. At 25 nM bombesin, an increase in $[Ca^{2+}]_o$ from 1 to 5 mM increased the percentage of cells which displayed repetitive Ca^{2+} transients. At 3 mM $Ca^{2+}{}_o$, repetitive transients induced by bombesin were completely inhibited (after one further transient) by the addition of EGTA or by the inhibition of Ca^{2+} inflow either by addition of verapamil or by activation of protein kinase C. Addition of Ca^{2+} to cells incubated in the absence of $Ca^{2+}{}_o$ and presence of bombesin immediately induced Ca^{2+} transients (50% of the cells tested). Thapsigargin (160 μM) induced a sustained increase in $[Ca^{2+}]_i$ while caffeine (10 mM) did not alter $[Ca^{2+}]_i$. Thapsigargin (4 cells) but not caffeine (8 cells) inhibited bombesin-induced Ca^{2+} transients when the agent was added before bombesin. The results show that a novel feature of the action of mitogens on individual NIH-3T3 fibroblasts is the heterogeneity of responses in increased $[Ca^{2+}]_i$ and the rapid termination of Ca^{2+} transients when Ca^{2+} inflow is inhibited.
Supported by the Anti-Cancer Foundation of the Universities of South Australia.

PROTEIN KINASE IN INITIATION AND INHIBITION OF GROWTH FACTOR-INDUCED MEIOTIC CELL DIVISION,
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With the use of Xenopus oocytes as a model cell division system:
1) *Insulin may initiate meiosis through PKC as* a) insulin induces an increase in DAG within minutes,b) staurosporine inhibits meiosis when added with insulin,c) at concentrations that block DAG production, neomycin inhibits insulin-induced meiosis. *PKC may be inhibitory to further progression of meiosis as* a) staurosporine, a PKC inhibitor, stimulates when added 1 hr after insulin,b)an agent which raises DAG and presumably stimulates PKC (PC-specific phospholipase C; PC-PLC), inhibits insulin-induced meiosis at about 1 hr,c) DAG levels again increase after this period. *Finally, PKC may again be involved in late events just before cell division (at the 6th hour)* as a) staurosporine addition at 3 hrs inhibits meiosis once again,b)there is a second peak of DAG at 5.1 hrs.
2) *Progesterone apparently does not initiate meiosis through a stimulation of PKC and PKC is inhibitory during this early period as* a) staurosporine stimulates meiosis when added with progesterone or about 1.5 hrs later (no effect if added much earlier or later),b)PC-PLC inhibits from steroid addition to about 1.5 hrs,c)progesterone induces a brief, rapid 20% decrease in DAG levels that is not noted with insulin,d)neomycin does not inhibit progesterone-induced meiosis. *PKC may be involved in a non-essential facilitation late in meiosis from about 1.5 to 3.6 hrs (cell division is 4 hrs after progesterone addition)* as a)PC-PLC addition at this time stimulates meiosis, b)progesterone induces a peak of DAG at 3.4 hrs,c)staurosporine does not inhibit meiosis when added during this period.

CHARACTERIZATION OF THE RECOMBINANT EXTRACELLULAR DOMAIN OF HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR EXPRESSED IN THE BACULOVIRUS SYSTEM, Pamela Brown, Suzanne Grothe and Maureen O'Connor-McCourt, Biotechnology Research Institute, National Research Council Canada, Montreal, Quebec, Canada H4P 2R2
In order to obtain large quantities of receptor protein for structural analysis, we have produced the extracellular domain of the epidermal growth factor receptor (EGFR-ED) in the baculovirus expression system. The DNA encoding the EGFR-ED was cloned into the transfer vector PJVETLZ behind the polyhedrin promotor. The recombinant construct was then incorporated by homologous recombination into the *Autographa californica* nuclear polyhedrosis virus genome. The host *Spodoptera frugiperda* Sf9 cells infected with the recombinant baculovirus produced the EGFR-ED which was detected 1) by Western blot using an anti-peptide antibody against a sequence in the extracellular domain, and 2) by an immuno-enzymatic assay using two anti-receptor monoclonal antibodies which are conformation dependent. The recombinant protein was present in both cell extracts and culture supernatants. The baculovirus-derived EGFR-ED was active since it could be specifically cross-linked to ^{125}I-EGF with BS^{3}.

IDENTIFICATION OF TWO TGF-β1 INDUCIBLE GENES WITH SHARED HOMOLOGY, A.M. Brunner, J. Chinn, M. Neubauer, L. Madisen, and A.F. Purchio. Oncogen, 3005 1st Ave, Seattle, WA 98121.

The transforming growth factor βs(TGF-β) are a family of highly related proteins that regulate cell growth and differentiation. To investigate TGF-β1's mechanisms of action, we have begun to identify genes whose expression is regulated by TGF-β1. A cDNA library was prepared from mouse embryo fibroblasts (AKR2B) treated with TGF-β1 in the presence of cyclohexamide for six hours. By differential screening, we isolated several clones which were either induced or repressed. Two clones, MEF-1 and MEF-2 will be discussed. The levels of these mRNAs increased following TGF-β1 treatment and DNA sequence analysis revealed that MEF-1 and MEF-2 are related to each other and to CEF-10, a gene induced by v-src in chicken embryo fibroblasts (Simmons et al. 1989, PNAS 86,1178). All three share >40% homology at the amino acid level and 38 of 39 cysteine residues are conserved. These data suggest that MEF-1 and MEF-2 may represent a new family of related proteins whose expression is modulated by TGF-β1, and thus, may also be involved in mediating the cellular response to TGF-β1.

T CELL RECEPTOR STIMULATION ACTIVATES A PROTEIN KINASE CASCADE. Victor Calvo*, Barbara E. Bierer*, R. L. Erikson^ and Terry Vik^ . *Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston , MA 02115 and ^ The Biological Laboratories, Harvard University, Cambridge MA 02138.
T cell receptor (TCR) stimulation activates multiple signal transduction pathways, including serine/threonine as well as tyrosine protein kinases. The 42 kDa MAP kinase has been shown to activate a 92 kDa S6 kinase upon insulin receptor stimulation. We have investigated this protein kinase cascade in the T cell line Jurkat. Stimulation via the TCR-CD3 complex with anti-CD3 MAb or incubation with PMA caused the rapid appearance of a 42 kDa phosphoprotein detected by anti-phosphotyrosine immunoblotting. The appearance of this tyrosine phosphoprotein correlated with activation of MAP kinase, assayed by the ability to phosphorylate recombinant S6 kinase, and with the activation of S6 kinase, assayed by the ability to phosphorylate S6-containing 40S ribosomal subunits. Both MAP kinase and S6 kinase activities, and the tyrosine phosphorylation of MAP kinase, were shown to be dependent on protein kinase C. Thus, we have demonstrated that the protein kinase cascade of MAP kinase activation resulting in S6 kinase activation is stimulated upon TCR triggering of T lymphocytes.

Growth Factor Signal Transduction

SYNTHETIC PEPTIDES DERIVED FROM $p34^{cdc2}$ ARE EFFICIENT PEPTIDE SUBSTRATES OF A $pp60^{c\text{-}src}$-RELATED PROTEIN TYROSINE KINASE, Heung-chin Cheng, Craig M.E. Litwin, David Hwang, and Jerry H. Wang, Cell Regulation Group, Department of Medical Biochemistry, University of Calgary, Calgary, AB, T2N 4N1, Canada.
A protein tyrosine kinase has been purified to near homogeneity from bovine spleen. The purified enzyme reacted immunologically with an antibody raised against a synthetic peptide corresponding to a highly conserved amino acid sequence (src(403-421)) of members of the $pp60^{c\text{-}src}$ family of tyrosine kinases. The spleen tyrosine kinase was found to efficiently phosphorylate a $p34^{cdc2}$-peptide, cdc2(1-24), which contained the regulatory tyrosine residue, TYR-15. In contrast, the peptide was a relatively poor substrate for EGF-receptor. Even though there are three tyrosine residues in cdc2(1-24), only TYR-15 was phosphorylated by the spleen tyrosine kinase. Studies of the phosphorylation of analogs of cdc2(1-24) by the spleen tyrosine kinase revealed GLU-12, the phenolic hydroxyl moiety of TYR-15, and a region located C-terminal of TYR-15 as structural motifs essential for efficient binding to and effective phosphorylation by the spleen tyrosine kinase.

POST-TRANSCRIPTIONAL CONTROL OF ALPHA 1 (I) PROCOLLAGEN IN *TRK* TRANSFORMED CELLS.M. Corominas, C.D. Hoemann and H. Zarbl. Division of Toxicology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

Two proteins expressed in Rat-1 cells which are targets for *v-fos* transformation-specific alterations in gene expression have been identified as $\alpha1$(I) and $\alpha2$(I) procollagen. Both proteins are present in Rat-1 fibroblasts but their synthesis is dramatically reduced after transformation with the FBJ-*v-fos* oncogene. Revertant cell lines, which express a functional Fos oncoprotein, resume synthesis of procollagen (I) at levels comparable to those seen in Rat-1 cells (Hoemann and Zarbl, Cell Growth and Differentiation, In press). A Western blot generated from whole cell extracts and probed with antisera specific for the C-terminal propeptides of $\alpha1$(I) and $\alpha2$(I) procollagen indicated that *v-Ha-ras*, *v-Ki-ras*, v-*fms*, *Erb B*, *mos*, *v-fes*, *sis*, *src* and polyoma middle T antigen failed to perturb the levels of procollagen either positively or negatively in Rat-1 cells. However, the *neu* oncogene reduced both $\alpha1$(I) and $\alpha2$(I) expression, and the *trk* oncogene abolished $\alpha1$(I) expression but only slightly reduced $\alpha2$(I) expression. Northern blot analysis and runoff transcription data indicate that the $\alpha1(I)$ procollagen is regulated at the post-transcriptional level in Rat-1 fibroblasts transformed with the *trk* oncogene. Moreover, $\alpha1$(I) procollagen mRNA in *trk* transformed cells is as stable as in the non-transformed cells and can be efficiently translated *in vitro* using the rabbit reticulocyte lysate. Further experiments on the postranscriptional processing and cellular localization of $\alpha1$(I) procollagen mRNA in *trk* transformed cells will be presented.

Overexpression of Mouse 4.5S RNA Correlates With Resistance to v-Ki-ras Transformation. M. L. Cutler, N. Talbot, and R. Bassin. Laboratory of Tumor Immunology and Biology, N.C.I., Bethesda, MD.

Using a cDNA expression cloning system designed to select genes capable of suppressing v-Ki-*ras* transformation of NIH-3T3 cells, a cDNA clone of mouse 4.5S RNA was isolated. Northern blot analysis revealed that this RNA is five to ten fold more abundant in the phenotypic *ras*- revertant cell lines C11, F2, and CHP-9CJ than in NIH-3T3 cells. These revertant cell lines,derived from v-Ki-*ras* transformed NIH-3T3 cells,are all resistant to transformation by v-*ras*. In addition, in comparison to uninfected cells there is a reduced level of this RNA in v-*ras*, v-*mos*, and v-*src* transformed NIH-3T3 cells. In both v-*ras* transformed and normal NIH-3T3 cells 4.5S transcripts are primarily nuclear in localization, sensitive to actinomycin D but not< -amanitin with a half life of approximately 30 minutes. However, in the ras revertant cell lines the higher level of 4.5S RNA can be partially accounted for by an increase in half life from 30 to 90 minutes. V-*ras* trans formed NIH-3T3 cells containing the 4.5S RNA cDNA clone transcribed from an RSV promoter appear morphologically flat, and are resistant to v-*ras*, v-*mos* and v-*src* transformation. These cells contain a long lived, vector derived nuclear transcript of the cDNA which may contribute to the suppression of the transformed phenotype. Current efforts are aimed at determining the role of this RNA in cell growth and transformation.

ISOLATION AND CHARACTERIZATION OF A GROWTH FACTOR-STIMULATED PROTEIN KINASE THAT PHOSPHORYLATES THE EGF RECEPTOR AT THR-669

Roger J. Davis, Ingrid C. Northwood and Fernando Gonzalez, Howard Hughes Medical Institute, Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA, 01605.

The epidermal growth factor (EGF) receptor is phosphorylated at Thr-669 after treatment of cells with EGF, platelet-derived growth factor, or phorbol ester. The mechanism of this increased phosphorylation is the stimulation of the activity of a protein kinase that has a substrate specificity for the EGF receptor at Thr-669. The sequence surrounding this phosphorylation site is Pro-Leu-Thr-Pro.

In order to understand the mechanism by which this protein kinase activity is regulated we have purified the enzyme to apparent homogeneity using FPLC procedures. The purified protein was digested with trypsin and the peptides obtained were examined by amino terminal sequence analysis. Comparison of the sequences obtained with Genbank (release 63.0) indicated no significant homology with any previously identified protein. Complementary DNA clones isolated from a human fibroblast library confirm the conclusion that this enzyme represents a novel growth factor-regulated protein kinase.

A MANGANESE-DEPENDENT SERINE KINASE COPURIFIES WITH PHOSPHOINOSITIDE 3-KINASE.

Christopher L. Carpenter, Brian C. Duckworth, Kurt R. Auger and Lewis C. Cantley. Department of Physiology, Tufts University Medical School, Boston, MA 02111.

Phosphoinositide 3-kinase (PI 3-kinase) is involved in growth factor and oncogene signal transduction. The enzyme is a heterodimer of an 85 kDa and one of two related 110 kDa proteins. In vivo the 85 kDa subunit of PI 3-kinase is phosphorylated on tyrosine, serine and threonine. In looking for serine/threonine kinases that phosphorylate PI 3-kinase we discovered that a serine kinase copurifies with PI 3-kinase. Native isoelectric focusing gels show that the two enzymatic activities are present in the same complex. The serine kinase activity is manganese dependent. It phosphorylates both subunits of PI 3-kinase and will phosphorylate casein and histone. There is no major effect of serine phosphorylation of purified PI 3-kinase on its enzymatic activity. Since only the two subunits of the PI 3-kinase are visible on silver-stained SDS-PAGE the serine kinase is either a minor contaminant or is one of the subunits of PI 3-kinase.

EGF RECEPTOR ENDOCYTOSIS: ENHANCED RATE OF INTERNALIZATION FOR HIGH AFFINITY, PHOSPHORYLATED RECEPTORS.

Stephen Felder, Jenifer LaVin, Axel Ullrich, & Yossi Schlessinger. Department of Cellular & Molecular Biology, Rorer Biotech. Inc., King of Prussia, PA. 19401.

High affinity EGF binding has been shown to be due to an elevated ON rate, that is EGF binds more rapidly to cells at low concentrations (Bellot et al., J. Cell Biology 110; 491, 1990). EGF is also internalized more rapidly at low concentrations than at high concentrations (Wiley et al., J. Cell Biology 107; 801, 1988). We demonstrate four ways to reduce the elevated rate of internalization at low EGF: treatment of cells with an antibody, treatment of cells with phorbol ester (two treatments which reduce high affinity EGF binding), point mutation at lys721 inactivating tyrosine kinase, or point mutation at thr654, a major protein kinase C phosphorylation site (two treatments not affecting high affinity binding). Hence, high affinity EGF binding, an active tyrosine kinase moiety, and an available thr654 phosphorylation site all appear necessary for elevated internalization.

Cell cycle dependent assembly and phosphorylation of replication initiation complexes. Rati Fotedar and James M. Roberts. Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104
Cell free replication of SV40 DNA was used as a model to understand the regulation of chromosome replication during the eukaryotic cell cycle. We have shown that initiation of DNA replication at the SV40 origin is cell cycle restricted - extracts from S phase cells actively promote initiation while extracts from G1 cells are inactive. Biochemical fractionation of extracts from human S phase cells identified a 250 kd multiprotein complex that could activate DNA replication when added to an extract from G1 cells. A necessary component of this complex is a human homolog of the S. pombe p34cdc2 kinase.

We have studied the structure and composition of replication initiation complexes assembled in vitro at the SV40 origin in order to understand the mechanism by which the p34 kinase regulates the start of replication. We have found that both the p34cdc2 protein and the helix destabilizing protein, RF-A, are tightly associated with the template DNA during the initiation of DNA replication. The interaction of these two proteins with DNA probably reflects the assembly of a functional initiation complex since their presence requires both the SV40 replication origin and the origin binding protein, SV40 T antigen. Moreover, this complex does not form in extracts from G1 cells directly demonstrating that the inability of G1 extracts to replicate DNA results from a failure to assemble the requisite proteins at the replication origin. The DNA associated p34 protein has protein kinase activity and will phosphorylate specific proteins, in cis, on the DNA as well as exogenously added histone H1.These studies have also revealed that the 33 kd subunit of RF-A becomes quantitatively phosphorylated within the initiation complex, while the free RF-A that does not participate in replication remains unphosphorylated. We are currently investigating the functional consequences of the phosphorylation of RF-A within the initiation complex.

PHOSPHORYLATION OF THE Thr669 SITE OF THE EGF RECEPTOR BYAN EGF-STIMULATED MAP KINASE FROM 3T3L1 CELLS, Irene Griswold-Prenner, Kunio Takishima, Thomas Ingebritsen and Marsha Rich Rosner, Ben May Institute and Dept. Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637 and Department of Zoology and Genetics, Iowa State University, Ames, IA 50011.
The epidermal growth factor (EGF) receptor is both an activator and a target of growth factor-stimulated kinases involved in cellular signalling. Residue threonine 669 (T699) of the EGF receptor is phosphorylated in response to a wide variety of growth modulating agents. MAP kinase is similarly phosphorylated as well as stimulated by growth activators. To determine whether a MAP-like kinase is responsible for T669 kinase activity in EGF-stimulated 3T3L1 cells, we partially purified and characterized the T669 kinase. The results suggest that a MAP kinase phosphorylates T669 and thus participates in a feedback loop, being activated by the EGF receptor and in turn phosphorylating the receptor. Supported by NIH grant CA-3554-1 to M.R.R.

A DEVELOPMENTALLY REGULATED cAMP INDUCIBLE DNA BINDING ACTIVITY THAT SPECIFICALLY RECOGNIZES THE CAEs OF THE PRESPORE SPECIFIC GENE *SP60* IN *D. DICTYOSTELIUM*,
Linda Haberstroh and Richard Firtel, Ctr. for Mol. Genetics., Dept. of Biology, UCSD, La Jolla CA 92093-0634

Previously, 5' deletion analysis has revealed 3 important CA rich sequence elements (CAEs) in the upstream region of the prespore gene SP60 of *D. discoidium*: consensus: CACACAnnnCACACA. Here, we have made site directed deletions and point mutations of these upstream elements. We see significant decreases in reporter gene activity (luciferase or *lac* Z) when one of the CAEs is deleted or mutated. A point mutation (mut D) within the middle CAE or deletion of the middle (2Δ1) or proximal (3Δ2) CAE results in substantial decreases in luciferase activity at 18 hr of development relative to the wild-type containing all 3 intact CAEs. *LacZ* fusions with all three mutants exhibit a homogeneous ß-gal staining pattern across the prespore region of the slug, which is less intense than that produced by wildtype *SP60/lacZ*. This contrasts the gradient staining pattern within the prespore zone that we previously observed with progressive 5' deletion of the CAEs. The endogenous *SP60* gene (ref. 1) and *SP60*/luciferase fusion gene (ref. 2) are induced by cAMP in shaking culture in the absence of multicellular development. Previous deletion analysis implicates CAES in cAMP inducibility of *SP60* expression.

We have used native polyacrylamide gels to identify an activity (CAE binding activity: CBA) from crude nuclear extracts that binds the CAE oligomers with varying affinity. CAE box 1 (most distal) binds the strongest; CAE box 2 (middle) binds with less affinity. All CAE oligomers cross compete for binding. This binding activity is present in nuclear extracts from cells in shaking culture induced by cAMP but not in those icubated without exogenous cAMP. A binding activity is induced during multicellular development with kinetics similar to that of *SP60* gene expression: it is not present in vegetative, detectable in 10hr, and strong in18hr extract and produces a shift at the same relative mobility as that for the cAMP inducible activity. An oligomer of the point mutant, mut D, neither binds nor competes for the cAMP, developmentally regulated factor(s).

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BIOCHEMICAL STUDIES OF THE PHEROMONE RESPONSE PATHWAY IN SACCHAROMYCES CEREVISIAE, Jodi E. Hirschman and Duane D. Jenness, Department of Molecular Genetics and Microbiology, University of Massachusetts Medical Center, Worcester, MA. 01655. In the budding yeast Saccharomyces cerevisiae, the transition from haploid to diploid life cycles is mediated by the binding of peptide pheromones to cell surface receptors. Pheromone binding causes arrest of cell division in the G1 phase of the cell cycle, followed by cell and nuclear fusion. The yeast receptor protein is a member of the β-adrenergic class of receptors which respond to extracellular signals via heterotrimeric G-proteins. Three of the genes known to function downstream of the receptor in the pheromone response pathway, SCG1, STE4, and STE18, show homolgy to the α, β, and γ subunits of heterotrimeric G proteins, respectively.

We have initiated biochemical studies to examine the functions of the SCG1, STE4, and STE18 gene products in vitro. To this end, we have raised polyclonal antisera to the SCG1 and STE4 proteins, using as antigen protein expressed in E. coli. We have constructed two yeast strains; the first expresses SCG1 at high levels, and the second overexpresses both STE4 and STE18. Each antisera recognizes a protein of the correct predicted molecular weight from crude membrane fractions prepared from these two overexpressing strains. Solubilization of each protein requires detergent extraction of the membranes, suggesting that SCG1 and STE4 proteins are membrane-associated. We are developing purification procedures and assays for protein function.

MOLECULAR CHARACTERIZATION OF A NOVEL, PUTATIVE GROWTH FACTOR RECEPTOR.

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The pivotal role which growth factor receptors play in the growth regulation of eukaryotic cells has focused attention on the search for new members of this important family of molecules. By applying degenerate oligonucleotides deduced from highly conserved motifs within the Protein Tyrosine Kinase (PTK) family in a PCR based approach (1), we have isolated a novel cDNA clone, RIK. On the basis of its deduced amino sequence, RIK exhibits hallmarks characteristic of members of the PTK receptor family, namely, a putative transmembrane domain, a distally located potential ATP binding site and a hypothetical glycosylation rich extra-cellular domain. Analysis of the protein in A431 cells, indicates that RIK is exclusively membrane associated, has a molecular mass of 160-180 kD and exhibits tyrosine kinase activity. Intriguingly, RIK's primary sequence diverges at key amino acid residues, located within the highly conserved catalytic domain region of the PTKs. This, coupled with the presence of a large serine/threonine rich juxtamembrane region, lack of a kinase "insert" domain and non-conservation of cysteine residues or Ig-like domain motifs, present in the extra-cellular regions of other Receptor Tyrosine Kinases (RTK), ascribes RIK as a member of a new class of RTK. Studies to ascertain the physiological role of this new receptor have commenced, involving targeted mutagenesis via homologous recombination of a mutant murine RIK construct in ES cells, concomitant with studies to isolate the putative ligand of this novel receptor protein.

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REGULATION OF RENAL PHOSPHOENOLPYRUVATE CARBOXYKINASE (PCK) AND GLUTAMINASE (GA) GENE EXPRESSION BY PROTEIN KINASE C AND CYCLOHEXIMIDE

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The addition of phorbol 12-myristate 13-acetate (PMA) causes a rapid and reversible decrease in the levels of PCK and GA mRNAs in renal LLC-PK-F^{+} epithelial cells. The repressive effect of PMA overrides the stimulatory effects of acidosis on PCK and GA gene expression and the inductive effect of cAMP on PCK mRNA. The action of various protein kinase inhibitors and a synthetic diacylglycerol analogue indicate that these effects are due to an activation of protein kinase C. In contrast, the addition of cycloheximide (CHX) increases the levels of both mRNAs and reverses the repressive effect of PMA. Treatment with puromycin causes an equivalent induction of PCK and GA mRNA levels, whereas concurrent treatment with actinomycin D completely abolishes the effects of CHX. These data indicate that both PCK and GA are controlled by two different repressors. This hypothesis is supported by the results of CAT assays performed using constructs containing 5' deletions of the PCK promoter, which show that the negative effect of PMA requires a cis-acting element that is located between -109bp and -68bp. The only element which has been mapped in this region of the PCK promoter is the CRE which confers basal and cAMP induced transcription of the PCK gene by alteration of the phosphorylation state of the binding protein, CREBP. It is suggested that CREBP may also be phosphorylated by protein kinase C which leads to a negative effect on transcription. The CAT assay will also be used to map the site of the CHX effect.

CHARACTERIZATION OF A NOVEL REGULATORY FACTOR FROM HTLV-TRANSFORMED T CELLS,
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HTLV is known to immortalize and transform T cells, and it is postulated that *tax*, a HTLV 1 gene, is involved in this process. *tax* regulates in trans the expression of HTLV, as well as various cellular genes, including IL-2, IL-2Rα, GM-CSF and c-*fos*. The Tax protein does not bind directly with DNA, suggesting that it functions indirectly through one or more host cell factors. We report here the identification and characterization of a unique cellular factor, TF-1, that binds specifically to the 3 *tax*-responsive imperfect 21-bp repeats (sites 1, 2 and 3) within the U3 region of the HTLV-I LTR. Purification of TF-1 from nuclear extracts of an HTLV-transformed T-cell line, SLB-I, by site 2 DNA-affinity chromatography revealed 2 predominant polypeptide species with approximate molecular weights of 43 and 48 kD. Further characterization of TF-1 by gel retardation assay indicates that it is abundantly present in HTLV-transformed T-cell lines, but virtually undetectable in T-cell lines not infected with HTLV. In addition, the level of TF-1 binding activity does not correlate with either the presence or absence of HTLV Tax protein. Functional studies, however, suggest that TF-1 is *tax*-responsive; thereby implying that Tax modulates the transcriptional activity of TF-1. Detailed binding studies indicate that TF-1 specifically recognizes nucleotides within the conserved part of the "core" sequence, TGACG, of the 21-bp repeats in the HTLV-I LTR. Nucleotides adjacent to this core sequence influence TF-1 binding, resulting in variable affinity to the 3 repeats (site 2>>site 1>site 3). Cis-acting elements containing the core or related sequences (e.g. CREB, ATF, and AP1 binding sites) also compete efficiently for TF-1. Taken together, these data are consistent with a hypothesis that TF-1 is a novel T-cell regulatory factor related to the CREB/ATF/AP1 family. The mechanism by which HTLV Tax modulates TF-1 transcriptional activity is under investigation.

***trk*C, A NOVEL MEMBER OF THE *trk* GENE FAMILY OF TYROSINE PROTEIN KINASE RECEPTORS.** F. Lamballe, R. Klein and M. Barbacid. Department of Molecular Biology, Bristol-Myers Squibb Research Institute, Princeton, NJ 08543.
We have previously identified two tyrosine protein kinase genes, designated *trk* and *trk*B, that code for putative neurogenic cell surface receptors. Recently, we reported that the mouse *trk*B locus codes for at least two classes of receptor-like molecules, only one of which, gp145^{trkB}, contains a tyrosine kinase domain (Klein *et al.*, Cell **61**: 647, 1990). The non-catalytic *trk*B protein, gp95^{trkB}, has the same extracellular and transmembrane domains as gp145^{trkB}, but exhibits a very short cytoplasmic tail. Whereas transcripts coding for the catalytic gp145^{trkB} molecule were found in the cerebral cortex and hippocampus, those responsible for the synthesis of gp95^{trkB} were found in the ependymal linings of the ventricles and in the choroid plexus. In order to search for the putative ligand(s) of the *trk* and *trk*B receptors we turned to a porcine system. The porcine *trk*B locus also codes for two receptors, only one of which contains a tyrosine kinase domain. The 23 amino acid long cytoplasmic domain of the non-catalytic gp95^{trkB} protein is identical to the corresponding mouse sequence suggesting that this cytoplasmic region has functional relevance. Screening of a porcine brain cDNA library with a probe derived from the tyrosine kinase domain of the human *trk* gene, led to the isolation of a third member of this gene family. This gene, designated *trk*C, codes for a tyrosine kinase receptor which shares 86% and 90% amino acid homology with the tyrosine kinase domain of the *trk* and *trk*B gene products, respectively. Its extracellular domain also shares 54% and 52% homology with these proteins, including nine of its twelve cysteine residues. Preliminary nucleotide sequence analysis revealed various *trk*C cDNA clones lacking a catalytic tyrosine kinase region. Thus, the *trk*C locus, may also encode catalytic and non-catalytic receptor-like molecules.

TWO TRANSFORMATION DEFECTIVE POLYOMA MIDDLE T MUTANTS ASSOCIATE WITH p85 PI 3-KINASE BUT DO NOT MAINTAIN ELEVATED PI 3-KINASE PRODUCTS IN VIVO.
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Polyomavirus transforming protein middle T has no intrinsic enzymatic activity, but it associates *in vivo* with a number of cellular proteins. Two of these middle T associated proteins, PI 3-kinase and pp60^{c-src}, appear to be activated by their association with middle T. The tyrosine kinase activity of pp60^{c-src} is significantly increased when associated with middle T and levels of PI 3-kinase products, PI(3,4)P_2 and PI(3,4,5)P_3, are constitutively elevated in middle T transformed cells. Analysis of middle T mutants which disrupt only the association of PI 3-kinase or PI 3-kinase and pp60^{c-src} show that association of these two activities with middle T is necessary for transformation. However, formation of a complete middle T complex in itself is not sufficient for transformation. Two middle T mutants, 248m and dl1015, contain both PI 3-kinase and pp60^{c-src} as well as all other known wild type middle T associated proteins, yet are transformation defective. Since neither mutant exhibits any apparent defect in the associated pp60^{c-src} tyrosine kinase activity, the effect of these mutations on PI 3-kinase was investigated. *In vitro* PI 3-kinase activity in immunoprecipitations of 248m, dl1015 and wild type middle T showed similar substrate specificities, and *in vitro* phosphorylation of p85 by middle T complex protein kinases was also similar. However, serum starved cells expressing either 248m or dl1015 had decreased levels of PI(3,4)P_2 and PI(3,4,5)P_3 when compared to serum starved cells expressing wild type middle T. These results identify a common biochemical defect in these mutants and support the hypothesis that constitutively elevated levels of PI 3-kinase products correlate with transformation.

PKC-L IS A NEW MEMBER OF THE PROTEIN KINASE C FAMILY, HAVING UNIQUE TISSUE DISTRIBUTION[Etta Livneh, Nina Bacher and Eva Berent, Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

A new human full length cDNA, coding for a protein kinase, related to the protein kinase C (PKC) gene family was isolated. Although this protein kinase shares some homologous sequences and structural features with the four members of the PKC family initially isolated α, βI, βII and γ, it exhibits more homology with the recently described "PKC-related" subfamily, encoded by the cDNAs ε, ε and ζ. The transcript for this gene product, termed PKC-L, is most abundant in lung, is less expressed in heart and skin, but has very low expression in brain. Thus, its tissue distribution is unique and different than that described for other mammalian members of the PKC gene family, their expression being enriched in brain tissues. The ability of PKC-L to bind phorbol-esters was revealed by introducing the cDNA into COS cells. Moreover, we could show that this interaction stimulates its intrinsic kinase activity. Using PKC-L specific antibodies we could demonstrate the expression of PKC-L in several human cell lines including A431 cells. Its cellular localization and role in the regulation of cell growth is currently being studied.

DISSECTION OF INTERFERON-γ SIGNAL TRANSDUCTION SYSTEM: ANALYSIS OF IN-VITRO GENERATED HeLa MUTANTS THAT ARE UNRESPONSIVE TO INTERFERON-γ.

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A sensitive mutagenesis system was developed to isolate HeLa mutants that are unable to express MHC class II antigens upon induction by interferon-γ. After one round of chemical mutagenesis and selection, eleven clones were isolated and expanded. None of these mutants expressed any class II proteins or messages upon induction by interferon-γ. When induction of MHC class I antigen was examined, three clones were found to respond to interferon-γ, while the remaining 8 clones were unresponsive. Other interferon-γ inducible genes were also examined. The class I inducible mutants expressed normal inducible level of IRF-1, 1-8, and 9-27 messages; while the class I unresponsive mutants were totally devoid of any expression. These results demonstrate that there are distinct regulatory pathways for interferon-γ induction of class I and class II genes.

Expression of a Truncated Tyrosine Phosphatase Leads to Failure of Cytokinesis and Asynchronous Entry of Resulting Syncytial Nuclei into Mitosis. R. L. Margolis*, P.R. Andreassen*, D.E. Cool**, N.K. Tonks+, E.H. Fischer+, and E.G. Krebs**, *Hutchinson Cancer Research Center, Seattle, WA 98104, **HHMI, and +Department of Biochemistry, University of Washington, Seattle, WA 98195.

BHK cells have been transfected with plasmids containing either a full-length T-cell protein tyrosine phosphatase gene or the gene truncated in its carboxyl-terminus domain (D.E. Cool et al. PNAS, in press). In vitro assay of cell lines containing either plasmid shows increased protein phosphatase activity. Cells with the truncated gene become markedly bi/multinucleate (>60%) due to a defect in cytokinesis, whereas cells with the full-length gene or the carrier plasmid alone maintain a low background of binucleate cells. We have studied the nature of this failure in cytokinesis and find that the plasma membrane frequently fails to furrow in telophase, despite normal midbody formation from the anaphase spindle components. The resulting multinucleate cells exhibit dramatic asynchrony among their syncytial nuclei. For example, condensed chromosomes will coexist in the same cytoplasm as interphase nuclei. In the most severely affected populations, we observe asynchrony approaching 80-90% of the syncytial cells. The evidence supports the conclusion that synchronization of nuclei in syncytia for entry into mitosis, and the induction of cytokinetic furrowing, occur through trans-activating signals that are down-regulated by the truncated gene's expression. Microtubules associated with individual nuclei also exhibit mitotic cycle asynchrony. That is, mitotic spindles coexist in the same cytoplasmic space with interphase microtubule arrays. These results are interesting in light of recent reports that $p34^{cdc2}$ induces mitotic rearrangement of microtubule arrays growing from centrosomes in vitro (F. Verde et al. Nature 343:233, 1990), and also that $p34^{cdc2}$ localizes to the centrosome in late G2 and mitotic cells (E. Bailly et al. EMBO 8:3985, 1989). As $p34^{cdc2}$ activation for mitosis requires tyrosine dephosphorylation (K. Gould and P. Nurse, Nature 342:39, 1989), our data suggest the transactivating signals may involve the tyrosine phosphorylation state of $p34^{cdc2}$. Such signals may exist to coordinate mitotic events throughout the cytoplasm of normally mononucleate cells.

REGULATION OF PHOSPHOLIPASE C BY A G PROTEIN IMMUNOLOGICALLY DISTINCT FROM G_s, G_o, and G_is, Thomas F.J. Martin and Judith A. Kowalchyk, Zoology Research Building, University of Wisconsin, Madison, WI 53706

Hormone stimulation of phospholipase C (PLC) in membranes is GTP-dependent, implying mediation by a G protein. For several receptors (TRH in GH_3 cells), regulation is cholera and pertussis toxin-insensitive, suggesting a role for a novel G_p protein. To characterize G_p, an in vitro reconstitution assay was developed. The GH_3 cell membrane PLC is reversibly extracted from membranes with 1.0M KCl and GTP[S]-regulated activity is evident only when the PLC is membrane-associated. Solubilized brain membrane PLC-beta also associates with PLC-deficient GH_3 cell membranes and exhibits GTP[S]-stimulated activity. In a further modification, proteins from PLC-deficient membranes were extracted in cholate. Phospholipid vesicles incorporated with the membrane proteins restored GTP[S]-stimulated activity to the solubilized PLC-beta. The membrane component responsible for restoration of GTP[S] stimulation was found to copurify with known heterotrimeric G proteins on 4 chromatographic columns. Immunoprecipitation studies, which utilized peptide-specific antibodies to known G proteins, demonstrated that membrane cholate extracts could be depleted by 99% for G_o, G_{i3} and G_s and by 96% for G_{i2} without affecting the G_p activity detected in the reconstitution assay. Purified beta-gamma subunits were found to inhibit the activity of G_p. It is concluded that the pertussis toxin-insensitive G_p of GH_3 cells is a novel heterotrimeric G protein.

ISOLATION AND CHARACTERIZATION OF INTESTINAL EPITHELIAL CELL CLONES DISPLAYING HYPERSENSITIVITY OR RESISTANCE TO GROWTH INHIBITION BY TRANSFORMING GROWTH FACTOR-BETA (TGFβ), Kathleen M. Mulder[1], Sheila L. Morris[1], Ho Gene Choi[1], Jill Ziman[2], and Patricia R. Segarini[2], Baylor College of Medicine, Houston, TX 77030[1] and Celtrix Laboratories, Palo Alto, CA 94303[2].

TGFβ inhibits the proliferation of untransformed rat intestinal epithelial cells (IEC-18), maintained in 10% serum, in a concentration-dependent fashion (IC_{50}>200pM). When the same cells are continually maintained in serum-free, chemically-defined medium, a greater responsiveness to growth inhibition by TGFβ is observed (IC_{50}=10pM; 43% increase in doubling time). The parental IEC-18 cells were chemically mutagenized with ethyl methanesulfonate (EMS) for 24h to achieve approximately 96% kill. Following removal of EMS, mutagenized colonies which arose during 8d of growth in the presence of TGFβ (40pM; added every third day) were pooled, passaged twice, and cloned by limiting dilution. In a monolayer proliferation assay, two of the clones appeared to be totally unresponsive to either $TGFβ_1$ or $TGFβ_2$. Both of these clones displayed TGFβ-specific binding similar to that observed for the parental cells, suggesting that the insensitivity to TGFβ was not due to an inability to specifically bind TGFβ. A third clone was isolated which appeared to be hypersensitive to both forms of TGFβ (IC_{50}=25-30pM). This clone displayed an increased doubling time in the presence of either form of TGFβ, as well as a several-fold increase in receptor number relative to the parental cells. Receptor cross-linking studies will enable determination of the relative levels of the functional receptors in the hypersensitive clone. Mechanisms underlying the hypersensitivity or resistance of the clones to TGFβ will be discussed.

EVIDENCE FOR POLYMERIC FORMS OF Gα-PROTEINS; A NEW VIEW OF THE DYNAMIC ACTIONS OF GTP AND HORMONES, Shun-Ichi Nakamura and Martin Rodbell, Section on Signal Transduction, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709.

We have reported(Proc.Natl.Acad.Sci.87, 6413-6417,1990) that octylglucoside (OG) extracts polydisperse structures of the major types of transducing GTP-binding proteins (α_s, α_i, α_o) from rat brain and liver membranes. Sedimentation velocity measurements on sucrose density gradients revealed that these structures have sedimentation constants in the range of ~5S to greater than 12S. These structures are not associated with the β-subunits normally considered to be linked to the α-subunits of heterotrimeric G proteins extracted with cholate or lubrol. When incubated with 10-100 μM GTPγS (or AlF4), the large structures are converted to much smaller structures (~4S) which are likely monomers or oligomers of the Gα proteins. Based on these findings, it was suggested that the large polydisperse structures represent polymers of the α-proteins and that the non-physiological activators of G proteins induce disaggregation of the polymers to monomers or oligomers. Recently we have found that peptide hormones, catecholamines and carbachol induce changes in the polymeric structures of the α-proteins present in liver and brain membranes. Hormone action requires membrane integrity and the presence of guanine nucleotides. However, in contrast to the complete disaggregation of the polymers to monomers observed with GTPγS, hormones decrease the size of the polymers. An hypothesis is presented which suggests that hormone receptors interact with polymeric forms of GTP-binding proteins in a manner similar to dynamic regulation of F-actin and microtubulin by their specific regulatory binding proteins.

PHORBOL ESTERS DOWN-REGULATE TRANSCRIPTION AND TRANSLATION OF THE CD4 GENE. Neudorf, S., Jones, M., Parker, S., Papes, R., Lattier, D. Divisions of Hematology/Oncology and Pulmonary Biology, Children's Hospital Medical Center, Cincinnati, OH. 45229.
Treatment of CD4+ cells with PMA results in transient modulation of CD4 expression with normalization of expression occurring by 48-72 hr. The relatively long delay in normalization of CD4 expression cannot be accounted for by receptor turnover alone and suggests that PMA may also affect transcription and/or biosynthesis of CD4. We therefore studied the effects of PMA on biosynthesis and transcription of the CD4 molecule and gene in order to define mechanisms of PMA-induced modulation of CD4 expression. Cells treated with PMA showed reduced biosynthesis of the CD4 molecule but not of Class-I HLA molecules. Furthermore, PMA treatment resulted in reduced steady state levels of CD4 mRNA and inhibition of the relative rate of transcription of the CD4 gene. Steady state levels of actin mRNA and the relative rate of transcription of the actin gene were not inhibited by PMA. Cells expressing transfected CD4 cDNA gene products modulated in response to PMA, however, re-expressed CD4 earlier than similarly treated cells expressing the product of the endogenous CD4 gene. These data suggest that the cell surface expression of the CD4 molecule is probably down-regulated at the level of the protein as well as the gene and that inhibition of transcription affect the kinetics of CD4 expression.

GENOMIC STRUCTURE OF THE FOS B GENE: DIFFERENTIAL SPLICING GIVES RISE TO TWO TRANSCRIPTS, Tetsuro Noguchi, Pedro Lazo, Karen Dorfman and Rodrigo Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543-4000.

Fos B was first identified in NIH3T3 cells as an immediate early gene encoding a 338 amino acid protein presenting a 70% similarity with *c-fos*. The kinetics of induction of *fos B* mRNA is very rapid and transient similar to that of *c-fos*, therefore it was of interest to characterize the gene structure of fos B in order to determine its possible similarity with the *c-fos* gene and to study the 5' upstream *cis*-elements involved in the control of its expression.

We have shown that the *fos B* gene consists of 4 exons and 3 introns, similar to the *c-fos* gene. The third exon of *fos B* which encodes 36 amino acids containing the basic region of the DNA binding domain presents a 90% identity with *c-fos*.

By deletion analysis we have characterized a region upstream of the TATA box which is likely to be the promoter region of the gene. Several consensus sequences have been identified, including an SRE and AP-1 binding site whose relative positions are identical to that in the 5' upstream region of the *c-fos* gene.

The *fos B* gene gives rise to two transcripts by alternative splicing of exon 4. The spliced shorter form of *fos B* (*fos B*/SF) generates a protein of 236 amino acids (FOS B/SF) which also form complexes *in vivo* with the JUN proteins. The biological activity of FOS B and FOS B/SF will be presented.

ANALYSIS OF PROTEINS THAT ASSOCIATE WITH THE MURINE IL-3 RECEPTOR (AIC2A), Toshiya Ogorochi and Atsushi Miyajima, Department of Molecular Biology, DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304-1104.
The mouse AIC-2A gene encodes a mature protein of 120 kDa which binds IL-3 with low affinity when expressed on COS7 cells (1). Evidence indicates that the AIC2A gene product is the major IL-3 binding component of the high affinity IL-3 receptor. To analyze molecules which may modulate the affinity of AIC2A and transduce signals, we raised three monoclonal antibodies specific for AIC2A protein (2). One of these antibodies, designated as 3D1, immunoprecipitated several proteins in addition to the AIC2A protein from IL-3 dependent cell line, PT18. These proteins were designated as p140, p120 (AIC2A), p90, p80, p75, p60 and p55 based on their mobility on reducing SDS-PAGE. Peptide mapping of proteins by V8 protease showed that p75 was a degradation product of p120, and p80 and p60 are distinct from p120. Western blotting using anti-phosphotyrosine antibody showed that p140, p75 and p55 were tyrosine phosphorylated. p120, p75 and p55 were recognized by the antibody (#191) raised against the specific peptide sequence of AIC2A. An *in vitro* kinase assay of the immunoprecipitate showed that p90, p80 and p60 were phosphorylated at serine and threonine residues. Phosphorylated p90 and p80 labelled *in vitro* showed different cleavage patterns by V8 protease. These results indicate that p90, p80 and p60 are unique proteins; characterization of these proteins will be presented.
(1) Itoh et al., (1990) Science, 247, 324, (2) Ogorochi et al., (1990) ASBMB/AAI Joint Meeting

NUCLEAR TRANSLOCATION OF THE CATALYTIC SUBUNIT OF PROTEIN KINASE A IS ESSENTIAL FOR cAMP-STIMULATED GENE TRANSCRIPTION.
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cAMP-regulated expression of many eukaryotic genes is mediated by cAMP-dependent protein kinase (PKA). The inactive PKA holoenzyme is activated when cAMP binds to its regulatory subunit, resulting in the release of active catalytic (C) subunit. Free C subunit in turn stimulates gene transcription by phosphorylation of the transcription factor CREB. Circumstantial evidence suggests that this phosphorylation is a nuclear event: CREB is localized in the nucleus, and cAMP-induced dissociation of the type II isozyme of PKA has been reported to result in translocation of the C subunit from the golgi to the nucleus. Recently, we have described a mutant regulatory subunit of the type II isozyme of PKAII that no longer inhibits catalytic activity towards the synthetic substrate kemptide but can still form holoenzyme. We report now that holoenzyme containing this mutant regulatory subunit phosphorylates physiological substrates, such as ribosomal S6 protein and CREB, *in vitro*. However, it does not stimulate gene transcription *in vivo*. In contrast to free C subunit the mutant holoenzyme is only found in the cytoplasm but not in the nucleus, as shown by immunocytochemitry. We conclude that physical separation of C subunit and CREB prevented phosphorylation of CREB, and that translocation of C subunit is essential for activation of gene transcription.

ISOLATION OF A NOVEL ONCOGENE (SHC) ENCODING A SH2 PROTEIN.
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Cytoplasmic tyrosine-kinases bear a non-catalytic domain, SH2, which is involved in the regulation of the kinase activity . The SH2 domain is also present in the ras GTPase-activating protein (GAP), the phospholipase C-148 and the crk oncogene product. We searched for novel SH2 sequences by screening a human B-lymphoid (P3HR1) cDNA library with a DNA probe representative of the c-fes SH2 domain and isolated a single cDNA clone (GF1). This clone was used as a probe for isolating eight more clones from a myeloid leukemia (KG-1) and a normal fetal brain cDNA library. Nucleotide sequencing revealed a 3031 bps novel sequence containing a open reading frame of 1419 bps, followed by an untranslated region of 1530 bps. The putative protein product is 473 aminoacids long, with a predicted molecular weight of 51 Kd. The novel gene is conserved in the evolutionary scale (from chicken to humans). The predicted protein is homologous to the SH2 domain and the amino-terminus of the protocollagen α1. In consequence the gene has been named SHC (from SH2 and collagen). Northern blot analysis disclosed variable amounts of two transcripts (3.7Kb and 4Kb) in most human tissues tested. The hematopoietic cells displayed only the 3.7Kb transcript. The role of the SHC gene in neoplastic transformation was investigated by transfecting NIH3T3 cells with the SHC gene driven by an RSV LTR. Costitutive expression of the gene leads to a transformed phenotype.

ACTIVATION OF THE PDGF RECEPTOR BY THE E5 TRANSFORMING PROTEIN OF BOVINE PAPILLOMAVIRUS,
Lisa Petti, Laura Nilson, and Daniel DiMaio, Department of Human Genetics, Yale University School of Medicine, New Haven, CT 06510.
The fibropapillomaviruses, such as bovine papillomavirus type 1 (BPV), induce tumors of fibroblasts and epithelial cells. The E5 gene is largely responsible for the ability of BPV to induce morphologic transformation of established rodent fibroblasts. E5 encodes a 44-amino acid, membrane-associated protein that is among the smallest documented transforming proteins. The E5 protein consists of two functional domains, a short "active site" at the carboxyl terminus containing seven amino acids which are essential for transformation, and a hydrophobic domain encompassing the amino terminal two thirds of the protein that can be replaced with certain random hydrophobic sequences. We have found that the β receptor for platelet-derived growth factor (PDGF) is constitutively activated in C127 and FR3T3 cells transformed by the E5 protein but not in untransformed cells or in cells transformed by a panel of other oncogenes. Receptor activation was demonstrated by an increase of tyrosine phosphorylation in vivo and by the presence of the receptor in a complex with increased tyrosine kinase activity as measured by an in vitro reaction. Receptor activation in response to acute E5-mediated transformation precedes induction of cellular DNA synthesis. Moreover, activation of the PDGF receptor by the E5 protein mimics the effects of PDGF in normal cells. On the basis of these results, we propose that the PDGF receptor is an important cellular transducer of the mitogenic signal of the E5 protein. There is a short region of sequence similarity between the active site of the E5 protein and PDGF, suggesting that the E5 protein may activate the PDGF receptor by binding directly to it.

Growth Factor Signal Transduction

POTENTIATION OF DNA-MEDIATED TRANSFECTION IN NIH3T3 CELLS BY ACTIVATORS OF PROTEIN KINASE C, James T. Reston, Dept. of Biological Sciences, Wadsworth Center for Laboratories and Research, Albany, N.Y., and Susan Gould-Fogerite, and Raphael J. Mannino. Department of Laboratory Medicine and Pathology, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103-2714
A growing body of evidence suggests that retroviruses utilize intracellular signal transduction pathways at various stages of their life cycle. The study of these mechanisms may aid in designing more efficient gene transfer vectors. A possible candidate for involvement in the transfer of viral DNA to the nucleus is the protein kinase C family of enzymes. As a starting point, the effects of PKC activators on the standard calcium-phosphate transfection technique were tested to see if any enhancement of transient and stable expression of transfected DNA could be observed. Addition of either TPA or DiC_8 to NIH3T3 cells 4 hrs. after DNA addition resulted in a 3-7 fold increase in the number of cells expressing transfected genes from two different plasmids, and a concomitant increase in the quantity of enzyme activity during periods of transient and stable expression. Treatment with the inactive phorbol ester analog aPD_2 failed to similarly enhance transfection efficiency, while the PKC inhibitor sphingosine blocked the TPA-mediated increases in expression levels. The results suggest that PKC activation enhances both transient and stable expression levels of transfected DNA in NIH3T3 cells.

TRUNCATION OF THE CYTOPLASMIC TAIL OF CD3 DELTA YIELDS UNALTERED T CELL RECEPTOR FUNCTION, Tanya M. Rutledge**, Barbara B. Niklinska*, Stuart J. Frank, Jonathan D. Ashwell* & Richard D. Klausner. Cell Biology and Metabolism Branch, NICHD, NIH, Bethesda, Md 20892. *Biological Response Modifiers Program, NCI,NIH, Bethesda, Md 20892. **HHMI-NIH Research Scholar.
The T cell receptor is a multisubunit complex composed of the antigen recognizing alpha-beta heterodimer, the CD3 complex and the zeta-zeta or zeta-eta dimer. Engagement of the T cell receptor in the T cell hybridoma line, 2B4, results in several biochemical and functional events. The presence of the zeta-eta heterodimer in the TCR correlates with phosphoinositide hydrolysis, PKC activity and apoptosis, while receptors with the zeta-zeta homodimer are sufficient to mediate tyrosine kinase activity, IL-2 production and growth inhibition. Antigen stimulation of the TCR requires zeta to transmit the state of receptor occupancy to other signalling molecules. We sought to determine whether elements of the CD3 complex, whose function is as yet unknown, might serve as this ultimate TCR signal transducer. To investigate a specific functional domain of the CD3 complex, cDNAs encoding truncations of the cytoplasmic tail of CD3 delta were cotransfected with a zeta cDNA into a 2B4 variant cell line , 3.12.29.19, that is zeta/eta negative, delta deficient and surface receptor negative. Cotransfection of full length and truncated delta with zeta restored normal TCR surface expression. The signalling capabilities of truncated delta TCRs were not impaired in comparison with full length control transfectants. We observed comparable levels of IL-2 production in both cell types in response to either antigen or crosslinking anti-TCR antibody. Growth inhibition in response to crosslinking antibody was also similar between truncated delta transfectants and full length controls. Thus, the cytoplasmic tail of the CD3 delta chain is not required for antigen or antibody induced IL-2 production or for growth inhibition by crosslinking antibody in zeta-zeta containing TCRs. Its potential role in zeta-eta TCR mediated signalling phenomena remains to be elucidated.

REL B: A GENE WITH SIMILARITY TO THE C-REL PROTOONCOGENE, Rolf-Peter Ryseck, Paulina Bull[1], Heather Macdonald-Bravo and Rodrigo Bravo, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543-4000; [1]Universidad Catolica de Chile, Dpto. Biologia Celular y Molecular, Casilla 114-D, Santiago, Chile.

Serum stimulation of quiescent fibroblast cells induces the expression of more than one hundred genes among which are several protooncogenes encoding for transcriptional factors including *c-jun*, *c-fos*, and *c-rel*. Here we describe a novel immediate early gene containing a region of high similarity with *c-rel*, which we have named *rel B*.

The 2.2 kb *rel B* mRNA encodes for a 528 amino acids protein with a molecular weight of 65 kd as confirmed *in vivo* by immunoprecipitation analysis of $[^{35}S]$-methionine labelled cell extracts. After the first 97 amino acids REL B contains a region with a similarity of 62 % to c-REL and 54 % to the recently described NFkB, spanning in total 269 amino acids. The C-terminal part of REL B shows no homology to any known protein. Transcriptional activation analysis of Gal4-*rel B* fusion proteins in yeast reveal that the last 150 amino acid containing region highly stimulates the expression of β-galactosidase, whereas the complete *rel B* fusion protein is only a weak activator. The N-terminal part including the region with similarity to c-REL shows no detectable transcriptional activity. A similar situation was found recently for c-REL.

An analysis of genomic clones of the *rel B* gene and comparison with the organisation of *c-rel* from turkey reveal that in the region of similarity the exon/intron pattern is conserved, showing not only a functional but also an evolutionary relationship between the two genes.

Possible interaction of REL B with c-REL and NFkB and binding to an NFkB recognition site are currently investigated.

PURIFICATION, cDNA SEQUENCE, CELLULAR LOCATION, AND PKC PHOSPHORYLATION OF A 33-kDa ANNEXIN PROTEIN FROM HYDRA VULGARIS. David D. Schlaepfer, Douglas A. Fisher, Hans R. Bode, Jay M. Jones, and Harry T. Haigler, University of California, Irvine, CA 92717.

Annexins are a family of Ca^{2+}-binding proteins that reversibly bind to specific phospholipids in a Ca^{2+}-dependent manner. Certain annexins (also known as lipocortins) are phosphorylated by growth-regulated tyrosine kinases and by PKC. The physiological function of annexins are not known so it is not yet possible to define the biological significance of the post-translational modifications. To investigate the evolution of the annexins and to find a simple system to elucidate function, we studied an annexin in the primitive freshwater coelenterate, *Hydra vulgaris.*

We purified an abundant 33-kDa protein (p33H) that had the Ca^{2+} and phospholipid binding properties expected for an annexin. P33H was phosphorylated by rat brain PKC *in vitro*. Amino terminal sequence analysis of p33H determined that it contained the annexin "Gly-Thr-Val-Lys" consensus PKC phosphorylation site. The nucleotide sequence of p33H cDNA showed that the protein had 44 to 53% amino acid sequence identity with the conserved core domains of mammalian annexins. Since p33H is not significantly more related to one mammalian annexin than another, it does not appear to be an invertebrate homologue of a previously characterized annexin. Affinity-purified polyclonal antibodies to p33H were used to determine its distribution in whole hydra by indirect immunoflourescence labeling. P33H showed a specific ectodermal epithelial cell expression pattern. P33H expression was maximal in differentiated epithelial cells of the tentacles, mouth, and foot regions of the animal. No detectable levels of p33H expression were found in nerve, endothelial, interstitial, or nematocyte (stinging) cells of the hydra. The specific epithelial cell expression pattern of p33H may indicate that this annexin is involved in the maintenance of hydra differentiated cell structures.

BOMBESIN IS A MITOGEN IN BALB/C 3T3 CELLS EXPRESSING A CLONED RECEPTOR FOR BOMBESIN/GRP. Thomas P. Segerson, S. Montgomery, E. Spindel. Vollum Institute, Portland, OR 97201 and Oregon Regional Primate Research Center, Beaverton, OR 97006.

Bombesin and its mammalian homologue gastrin-releasing peptide (GRP) are mitogenic in fibroblasts and some neuroendocrine cell lines. Our laboratories have cloned a cDNA for the bombesin/GRP receptor (B/GRP-R) by expression of a Swiss 3T3 cell library in *Xenopus* oocytes. To study the growth effects of bombesin peptides, we have introduced the B/GRP-R into heterologous mammalian cells. The B/GRP-R cDNA was expressed in Balb/c 3T3 cell lines using viral promotors and clones were selected for neomycin resistance. Resistant clones were screened for expression of B/GRP-R by [^{125}I]GRP binding assay. Two clones (Balb pLJBR 8 and Balb pLJ 18) which demonstrated high levels of [^{125}I]GRP binding were expanded and evaluated for their ability to initiate cell division after bombesin treatment. Quiescent cells were treated with 10 nM bombesin and loaded after 18 hours with either [^{3}H]thymidine or bromodeoxyuridine (BDUr) followed by evaluation at 22 hours for acid-precipitable radioactivity by scintillation counting or for chromatin-associated BDUr by immunohistochemistry using a monoclonal antibody to BDUr . Bombesin treatment increased the acid-precipitable radioactivity 4 to 5 fold, compared to no increase in untransfected Balb/c 3T3 cells. In bombesin-treated cells of the Balb pLJ 8 clone but not in untreated cells of this clone or treated wild-type cells, staining of chromatin-associated BDUr was markedly increased. We have developed a model to study the interaction of the bombesin receptor in cells in which this peptide is mitogenic. We are developing antibodies to the B/GRP-R and have introduced epitopes into the cloned receptor protein to study the interacations of this receptor which are responsible for its mitogenic activity.

Intracellular thiols in NF-κB activation and HIV transcription.

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The activation of NF-κB has been implicated in the regulation of transcription of a variety of genes (Il-2, Il-4, Il-6, TNFα) and has been shown to be essential for the expression of genes controlled by the HIV LTR. Intracellular thiols play a key role in this process, in that: 1) stimulation with TNFα, Il-1 and/or PMA activates NF-κB and markedly decreases intracellular thiols, 2) N-Acetyl L-cysteine, a glutathione precursor, prevents this thiol decrease and blocks the activation of NF-κB, 3) the lack of activated NF-κB prevents the activation of the HIV LTR and the transcription of genes under its control. PMA and TNFα show synergy in their activation of both NF-κB and the HIV LTR, as do PMA and IL-1. On the other hand, Il-1 and TNFα do not synergize and show the same degree of inhibition by thiols or the protein kinase inhibitors H-7 and H-9. This suggests that IL-1 and TNFα use the same signal transduction pathway, which is different from the PMA pathway.

These findings show a new type of regulation of NF-κB activity and HIV transcription, in which changes in intracellular thiol levels influence the activation of genes that are under the control of NF-κB and the HIV LTR.

Growth Factor Signal Transduction

THE GLYCOSYL PHOSPHATIDYLINOSITOL ANCHOR IS CRITICAL FOR LY-6A/E MEDIATED T CELL ACTIVATION, Bing Su, Gerald L. Waneck#, Richard A. Flavell, and Alfred L.M. Bothwell, Section of Immunobiology and Howard Hughes Medical Institute at Yale University Medical School, New Haven, CT 06510. # Surgical Services and MGH Cancer Center, Massachusetts General Hospital, Department of Surgery, Harvard Medical School, Boston, MA 02114

Ly-6E, a glycosyl phosphatidylinositol(GPI)-anchored murine alloantigen which can activate T cells upon antibody crosslinking, has been converted into an integral membrane protein by gene fusion. This fusion product, designated Ly-6ED^b, was characterized in transiently transfected COS cells and demonstrated to be an integral cell surface membrane protein. Furthermore, the fusion antigen can be expressed on the surface of the BW5147 class "E" mutant cell line, which only expresses integral membrane proteins but not GPI anchored proteins. The capability of this fusion antigen to activate T cells was examined by gene transfer studies in D10G4.1, a type 2 T cell helper clone. When transfected into D10 cell, the GPI-anchored Ly-6E antigen, as well as the endogenous GPI-anchored Ly-6A antigen, can initiate T cell activation upon antibody cross-linking. In contrast, the transmembrane anchored Ly-6ED^b antigen was unable to mediate T cell activation. Our results demonstrate that the GPI-anchor is critical to Ly-6A/E mediated T cell activation.

A 34 kD GTP-BINDING PROTEIN IS ASSOCIATED WITH THE p56^{lck}:CD4/CD8 T-CELL RECEPTOR COMPLEXES, Janice C. Telfer and Christopher E. Rudd, Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney Street and Department of Pathology, Harvard Medical School, Boston, MA 02115

In T-cells, GTP-binding proteins have been indirectly implicated, by cholera toxin inhibition and by G-protein activating molecules, as mediators between TcR:CD3 activation and subsequent phosphatidylinositol turnover. A major question has been to identify the GTP-binding protein and associated receptor involved in activation. The T-cell CD4:p56lck and CD8:p56lck receptor complexes include a 33-34 kD phosphoprotein. A pan-reactive anti-G-protein antisera specific for the consensus GTP-binding region Gly-$(Xaa)_4$-Gly-Lys recognizes a 33-34 kD phosphoprotein(p34). P34 is coprecipitated by anti-CD4, anti-CD8, and anti-lck antibodies from solubilized γ-^{32}P-labelled membranes. Immunoprecipitates formed by anti-CD4, and anti-CD8 antibodies bind GTP, and display GTP hydrolysis intermediate to that of purified G_iG_o and ras immunoprecipitated from T-cells. A receptor: tyrosine kinase: G-protein complex in T-cells could be of vital importance in the understanding of T-cell activation, autoimmunity, and oncogenesis.

GROWTH HORMONE STIMULATES TYROSINE PHOSPHORYLATION IN 3T3-F442A CELLS. Linda A. Winston, Meei-Lih Lin, and Paul J. Bertics, Department of Physiological Chemistry, University of Wisconsin, Madison, WI 53706

Growth hormone (GH) is known to stimulate a variety of biological responses in numerous cell types. A recent study found that GH inhibits the mitogenic effects of serum in 3T3-F442A cells, a cell line in which GH is believed to also have a role in adipogenesis. While the mechanism through which the GH signal is transduced is not well understood, a recent report suggested that the GH receptor is associated with tyrosine kinase activity. In the present study, the effect of GH on tyrosine phosphorylation was evaluated by probing lysates from recombinant GH-treated 3T3-F442A cells with anti-phosphotyrosine antibodies. GH treatment increased the amount of phosphotyrosine associated with at least two protein bands with molecular weights of approximately 90,000 and 100,000 in a GH concentration- and time-dependent manner, with maximal increases at 10 nM and 5 min, respectively. Preliminary evidence suggests that these effects of GH are not directly mediated by protein kinase C. Studies are presently underway to determine whether the GH-stimulated tyrosyl phosphoproteins are GH binding proteins.

A POTENTIAL ROLE FOR RAF-1 IN NEURONAL GROWTH FACTOR INDUCED DIFFERENTIATION OF PC12 CELLS, Kenneth W. Wood[1], Simon Halegoua[2] & Thomas M. Roberts[1] ; [1]Dana-Farber Cancer Institute, Boston, MA 02115; [2]Department of Neurobiology and Behavior, SUNY at Stony Brook, Stony Brook, New York 11794

The rat pheochromocytoma cell line PC12 differentiates in response to nerve growth factor (NGF) and fibroblast growth factor (FGF) to acquire the phenotype of a sympathetic neuron, including extended neurites. The molecular mechanisms by which these neuronal growth factors exert their effects have not been elucidated. Expression of both activated *src* and *ras* oncogenes induces differentiation of PC12, and their normal cellular counterparts are required for NGF and FGF action. The serine/threonine kinase Raf-1 has been identified as a potential downstream effector for a variety of receptors on murine fibroblasts. Raf-1 is phosphorylated in fibroblasts in response to phorbol ester, aFGF and PDGF treatment as well as in response to transformation by *v-src* . PDGF induced phosphorylation of Raf-1 is primarily on serine residues, though a small amount of tyrosine phosphorylation is detectable. We have investigated the potential role of Raf-1 in the response of PC12 cells to NGF and FGF as well to other growth and differentiation factors. Infection of PC12 cells with a retrovirus bearing an activated raf oncogene causes the extension of neurites, suggesting that Raf-1 may be another protooncogene component of the NGF signalling pathway for differentiation. In parallel experiments we have found that both NGF and aFGF treatment of PC12 cells results in the phosphorylation of Raf-1, primarily on serine residues. Raf-1 phosphorylation is apparently insufficient to induce differentiation; EGF and phorbol ester, both non-differentiating agents, cause Raf-1 phosphorylation. However, it has not yet been determined that the residues on Raf-1 phosphorylated in response to each of these factors are identical. While the majority of agents tested induced Raf-1 phosphorylation, PKA agonists and insulin did not. We are currently investigating the effect of these phosphorylations on Raf-1 kinase activity as well as the nature of activated Raf-1 induced differentiation of PC12 cells.