POSITIVE GROWTH CONTROL

Organizers: Charles Sherr, Thomas Curran, and Tony Hunter

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Poster Sessions

The Cell Cycle (Joint)

H 001 Mos PROTO-ONCOGENE AND CELL CYCLE REGULATION. George F. Vande Woude, Renping Zhou, Richard S. Paules[1], Ira Daar, Nelson Yew, and Marianne Oskarsson. ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702; [1]National Institute of Environmental Health Sciences, Mammalian Molecular Genetics Group, Research Triangle Park, NC 27709.

The mos proto-oncogene product, pp39^{mos}, is required for meiotic maturation in vertebrate oocytes and is an active component of cytostatic factor (CSF), an activity in unfertilized amphibian eggs believed to be responsible for their arrest at metaphase II. First described 20 years ago, CSF was shown to arrest egg development at M-phase by stabilizing high levels of maturation promoting factor (MPF). Thus, the mos product functions at a major cell cycle control point, and its activity is directly or indirectly responsible for the stabilization of MPF. This link between proto-oncogene function and M-phase cell cycle regulation could be responsible for certain phenotypes of transformed cells.

We have found that mos product is associated with and phosphorylates tubulin in vitro. Our analyses have also shown that β-tubulin is preferentially associated with and phosphorylated by the Xenopus mos product from either transformed cells or unfertilized eggs. Immunofluorescence and immunoelectron microscopy analyses have shown that the mos product in transformed cells co-localizes with tubulin in microtubules as well as in the metaphase spindle pole and early telophase mid-body and asters. We speculate that the mos product may contribute to the formation of the spindle pole as well as the spindle and thereby contribute (as CSF) to metaphase arrest. Constitutive expression of mos in somatic cells is sufficient for morphological transformation, but only cells expressing low levels of pp39^{mos} can grow as transformed cells. We postulate that this amount of product is not sufficient to cause mitotic arrest but is enough to impart M-phase phenotypes during interphase. Altered cell morphology and loss of contact inhibition during interphase could be due to cytoskeletal changes that normally occur during mitosis.

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Growth Factors

H 002 PLATELET-DERIVED GROWTH FACTOR: ISOFORM-SPECIFIC SIGNALLING VIA DIMERIC COMPLEXES OF TWO DIFFERENT RECEPTOR TYPES. Carl-Henrik Heldin*, Sejiro Mori*, Anders Eriksson*, Arne Östman*, Bengt Westermark# and Lena Claesson-Welsh*, *Ludwig Institute for Cancer Research, Box 595, BMC, S-751 24 Uppsala, Sweden, #Department of Pathology, University Hospital, S-751 85 Uppsala, Sweden.

Platelet-derived growth factor (PDGF) is a major mitogen for connective tissue cells and is composed of two disulphide-bonded homologous polypeptide chains, denoted A and B. Apart from being a platelet release product, PDGF has been found to be produced by several normal as well as malignant cell types, and has therefore been implicated in autocrine and paracrine growth stimulation. The three dimeric PDGF isoforms exert their functions via interaction with two distinct receptor types denoted α and β. The receptors are structurally similar; both are protein tyrosine kinases with characteristic insert sequences in their kinase domains, and have extracellular parts consisting of five immunoglobulin-like domains. Both α- and β-receptors undergo dimerization after ligand binding, which activate the receptor kinases. The dimerization occurs via binding of each subunit in the dimeric PDGF molecule to one receptor molecule; the A-chain of PDGF binds to α-receptors, whereas the B-chain of PDGF binds to both α- and β-receptors. PDGF-AB acts as β-receptor agonist in the presence of α-receptors, but as a β-receptor antagonist in the absence of α-receptors, which suggests that it activates the β-receptor via formation of heterodimeric receptor complexes. Thus, depending on PDGF isoform, homodimeric or heterodimeric receptor complexes will be formed.

In order to determine structural determinants in the PDGF B-chain that are involved in binding to the β-receptor, amino acid residues in the B-chain were systematically replaced by the corresponding residues in the A-chain which can not bind to the β-receptor. By this approach, three amino acid residues in the B-chain (Asn-115, Arg-154 and Ile-158) were identified to be of importance for β-receptor binding. A similar approach was used to demonstrate that a basic motif in the C-terminus of the B-chain causes retention of a major part of the B-chain in the Golgi complex. This motif is not present in the common form of the A-chain, but is found in a longer splice variant of the A-chain, which also is retained by the producer cell.

The structural and functional properties of the PDGF α- and β-receptors are currently being analyzed by expression of wild type and mutated cDNAs in porcine aortic endothelial cells, which lack endogenous PDGF receptors. A point mutated kinase negative β-receptor mutant is unable to mediate growth, chemotaxis or actin reorganization, and shows a decreased rate of internalization and degradation in response to ligand binding. Analysis of a series of C-terminally truncated receptor mutants has furthermore led to the identification of a hydrophobic sequence just C-terminal of the kinase domain which is of importance for ligand-induced receptor internalization. Ligand binding induces polyubiquitination of the β-receptor. Polyubiquitination is not seen in the kinase negative mutant of the receptor or in a C-terminally truncated receptor. The possible functional importance of polyubiquitination in receptor metabolism will be discussed.

Whereas both PDGF α- and β-receptors were found to transduce mitogenic signals, only the β-receptor mediated actin reorganization and chemotaxis. Analysis of the pattern of kinase substrates for the two receptors revealed similarities as well as differences; substrates phosphorylated by the β-receptor, but not by the α-receptor, may be involved in stimulation of chemotaxis and actin reorganization.

H 003 CSF-1 ACTION. E. Richard Stanley, Manuela Baccarini, Persio Dello Sbarba, Wei Li, Jeffrey W. Pollard, Y.G. Yeung, Albert Einstein College of Medicine, Bronx, New York 10461

Colony stimulating factor-1 (CSF-1) regulates mononuclear phagocyte survival proliferation and differentiation.[1] Several recent studies in this laboratory have focused on the changes that take place within the macrophage with the first 30 seconds of CSF-1 binding at 37°C. These changes include rapid non-covalent dimerization (165 kDa → 380 kDa) and tyrosine phosphorylation of the CSF-1 receptor, tyrosine phosphorylation of cytoplasmic proteins, covalent linking of receptor dimers by disulfide bonds, further receptor tyrosine phosphorylation and increased receptor serine phosphorylation as well as a covalent modification of one of the receptor subunits of the 380 kDa dimer that causes an apparent change in subunit molecular weight from 165 kDa to ~250 kDa.[2,3] The kinetic relationships between these changes have been elucidated by studies carried out at 4°C where the rates of all these events are slowed. Experiments have been undertaken to determine whether any of the cytoplasmic proteins that are rapidly phosphorylated in tyrosine (115, 99, 86, 75, 57, and 36 kDa) might be involved in a common post-receptor pathway regulating entry of cells into S-phase. In macrophages stimulated with CSF-1 or granulocyte-macrophage CSF, or in fibroblasts stimulated with platelet derived growth factor only one of these proteins (57 kDa) exhibits increased phosphorylation in tyrosine in all cell and growth factor combinations.[4] Studies on the nature of the 57 kDa protein are currently in progress. Among 3/20 independently arising mutagen induced CSF-1 independent mouse macrophage cell line mutants, proteins of M_r 135, 86 and 57 kDa, whose tyrosine phosphorylation is normally induced by CSF-1, are tyrosine phosphorylated in the absence of growth factor.[5] Recent studies on the CSF-1 biology and signal transduction will be discussed.

1. Stanley, E.R. (1990) in "Genetics of Pattern Formation and Growth Control" Wiley-Liss, N.Y. pp.165-180.
2. Li, W. and Stanley, E.R. (1991) EMBO J. 10:277-288.
3. Baccarini, M., Li, W., Dello Sbarba, P. and Stanley, E.R. (1991) Receptor, In Press.
4. Li, W., Yeung, Y.G. and Stanley, E.R. (1991) J. Biol. Chem. 266:6808-6814.
5. Dello Sbarba, P., Pollard, J.W. and Stanley, E.R. (1991) Growth Factors 5:75-85.

Receptors I

H 004 MOLECULAR BIOLOGY OF THE GM-CSF RECEPTOR, Nicholas M Gough, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia.

Eighteen distinct haemopoietic growth factors have now been cloned and characterized. The biological activities of these cytokines are for the most part characterized by their pleiotropy (action on several different type and at different levels) and redundancy (several different cytokines have similar actions). An understanding of these complex actions and interactions in regulating cell growth and differentiation requires an understanding of the biochemistry and molecular biology of the receptors on target cells with which these cytokines interact. Haemopoietic growth factor receptors generally exist in both a high and low-affinity form on target cells. In the case of GM-CSF, a glycoprotein which stimulates the proliferation, differentiation and functional activation of granulocytes and macrophages, receptors of both high (K_D ~30pM) and low (K_D ~1nM) affinity have been detected and chemical cross-linking studies have revealed multiple molecular species in the range 50,000-180,000 Daltons. The higher molecular weight species have been correlated with high affinity binding and the lower molecular weight species with low affinity binding. Binding of GM-CSF to its receptor can be modulated or competed by interleukin 3 under certain circumstances. We have cloned a cDNA specifying the primary chain of the human GM-CSF receptor (GM-Rα), a polypeptide of 378 residues with low binding affinity. The arrangement of four of 11 cytokine residues in the extracellular domain, as well as several other stretches of conserved sequence, notably the motif Trp-Ser-X-Trp-Ser identified the GM-CSF receptor α chain as a member of a newly recognized family of growth factor receptors, now known to encompass receptors for GM-CSF, IL-3, IL-5, IL-2 (β chain), IL-4, IL-6, IL-7, erythropoietin, G-CSF, LIF, growth hormone, prolactin, ciliary neurotrophic factor and the proto-oncogene c-mpl. Recently a second chain of the GM-CSF receptor complex (GM-R β), which confers high binding affinity, has been cloned and is also a member of this family The gene for the α chain of the human GM-CSF receptor has been localized to the short arm of both the X and Y chromosomes, within the pseudoautosomal region, a region of ~2.6 mbp of homologous DNA localized at the tip of the sex chromosomes which recombines during male meiosis. The GM-R α chain gene has been located 1100-1300 kbp from the telomere. The GM-R β chain gene has been localized to 22q 12.3-13.1. In this presentation, the molecular biology and biochemistry of the haemopoietin family of receptors will be discussed, with particular emphasis on recent results from our laboratory on the human GM-CSF receptor.

H 005 SIGNAL TRANSDUCTION BY IL-2 RECEPTOR, Tadatsugu Taniguchi[1], Masanori Hatakeyama[1], Takeshi Kono[1], Naoki Kobayashi[1], Seijiro Minamoto[1], Atsuo Kawahara[1], Steven D. Levin[2], Roger M. Perlmutter[2], [1]Institute for Molecular and Cellular Biology, Osaka University, JAPAN, [2]Howard Hughes Medical Institute, University of Washington, School of Medicine, USA.

Interleukin-2 (IL-2) has been known as a critical regulator of lymphocyte proliferation. The IL-2 receptor (IL-2R) comprises at least two distinct IL-2 binding components, the IL-2Rα chain (p55, Tac-antigen) and IL-2Rß chain (p70-75). Structural and functional analyses of the two components revealed that (i) IL-2Rα and IL-2Rß bind IL-2 with "low" and "intermediate" affinities (Kd: ~10nM and 1nM); respectively, and the two chains constitute the "high-affinity" IL-2 receptor (Kd: ~10pM); (ii)IL-2Rß but not IL-2Rα is responsible for the intracellular signal transduction; (iii) IL-2Rß belongs to a new superfamily of cytokines receptors. Detailed analysis of structural domains of the human IL-2Rß chain has revealed a critical cytoplasmic region rich in serine residues ("serine-rich" region) proximal to the cell membrane, important for ligand-mediated growth signal in an IL-3-dependent cell line, BAF-B03. Adjacent to this region, there is a region rich in acidic amino acids ("acidic" region). Both of these regions are highly conserved in human and murine IL-2Rß. However, the cytoplasmic domain of IL-2Rß does not contain any known catalytic domain, suggesting the possible involvement of other cellular component(s) interacting with the above-mentioned cytoplasmic domain.

We have evidence for the formation of a stable complex of IL-2Rß with the lymphocyte specific protein tyrosine kinase $p56^{lck}$. As a result of this interaction, IL-2Rß becomes phosphorylated *in vitro* by $p56^{lck}$. Treatment of T lymphocytes with IL-2 promotes $p56^{lck}$ kinase activity. These data suggest the participation of $p56^{lck}$ as a critical signaling molecule downstream of IL-2R via a novel interaction. These findings are also suggestive of the involvement of *src*-family tyrosine kinases in IL-2 and other cytokine mediated signals.

Work is in progress to identify the genes for cyclins and (cdc2-type) kinases which are induced by IL-2 and related cytokines.

Receptors II

H 006 SIGNALLING THROUGH THE PLATELET-DERIVED GROWTH FACTOR BETA-RECEPTOR, Jonathan A. Cooper, Adam Kashishian, Andrius Kazlauskas[1], Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, Washington 98104, and [1]National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, Colorado 80206.

PDGF binding stimulates PDGF receptor kinase activity, and induces tyrosine phosphorylation of the receptor and cell proteins. Protein substrates include a phosphatidyl-inositol (PI) 3 kinase, the Ras GTPase activator protein (GAP) and phospholipase C (PLC) γI, which are believed to be involved in signal transduction.

Phosphorylation of the human PDGF β-receptor at Tyr 857 is important for full kinase activity. Mutation of Tyr 857 to Phe reduces the in vivo phosphorylation of cellular substrates, the in vitro kinase activity of the receptor, and mitogenic signalling. Other tyrosine phosphorylation sites have been mapped to the kinase insert, a non-catalytic region within the kinase domain, and to the C-terminal tail. Mutant receptors in which each of the kinase insert tyrosines are mutated to phenylalanine have been expressed in dog epithelial cells. Peptide mapping of these receptors shows that Tyr 740, 751 and 771 are phosphorylation sites in vivo and in vitro. These mutants show specific defects in binding of intracellular proteins.

With wild-type PDGF receptors, PDGF stimulation induces the binding of several proteins that contain SH2 domains, including PI3 kinase, GAP, PLCγ, and Src. In vitro studies indicate that phosphorylation of the receptor creates binding sites for these proteins. We have studied the binding of PI3 kinase and GAP to each of the mutant receptors. Most of the Tyr to Phe mutations in the kinase insert have no effect on binding of either protein, but mutation of Tyr 740 inhibits binding of PI3 kinase, mutation of Tyr 771 inhibits binding of GAP, and mutation of Tyr 751 reduces binding of PI3 kinase when receptors are expressed at low, but not high, levels. These specificities are also evident in binding studies done with immunoprecipitated, phosphorylated receptors and purified or crude PI3 kinase or GAP.

These studies suggest that individual SH2-containing proteins distinguish phosphotyrosines lying in different sequence environments. GAP binds to P.Tyr 771 whereas PI3 kinase binds to P.Tyr740. In addition, P.Tyr 751 is required for PI3 kinase binding when receptors are expressed at low level. This indicates that high affinity binding of PI3 kinase requires two phosphorylated residues, but low affinity binding requires only one. Two phosphorylated residues may be needed to accommodate the two SH2 domains of PI3 kinase. Which SH2 domain interacts with which P.Tyr is presently being studied.

H 007 SH2 DOMAINS CONTROL INTERACTIONS OF TYROSINE KINASES WITH THEIR TARGETS. Tony Pawson, Xingquan Liu, Jane McGlade, Christine Ellis, Michael Reedijk, Luc Marengere, Gerry Gish and Andrew Laudano*. Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, 600 University Avenue, Toronto, Ont. M5G 1X5, Canada; *Department of Biochemistry, University fo New Hampshire, Durham, NH 03824.

Src homology (SH) regions 2 and 3 are noncatalytic domains that are conserved among a series of cytoplasmic signalling proteins, including Ras GTPase activating protein, phospholipase C-γ, phosphatidylinositol (PI) 3'-kinase, and Src. The SH2 domains of these signalling proteins bind tyrosine phosphorylated polypeptides implicated in normal signalling and cellular transformiation. Tyrosine phosphorylation acts as a switch to induce the binding of SH2 domains, thereby mediating the formation of heteromeric complexes at or near the plasma membrane.

Autophosphorylation of growth factor receptors induces high affinity binding of several SH2-containing proteins, which are likely targets of receptor tyrosine kinase activity. Different receptors show distinct binding specificities for SH2-containing signalling molecules such as PLC-γ1 and GAP. In some cases, the isolated SH2 domains of these proteins bind to activated receptors in vitro with the same specificity as do the intact native proteins in vivo. Hence, there is some specificity in the interactions of SH2 domains with receptors. The affinities with which different SH2 domains and autophosphorylated receptors associate may therefore control the activation of specific signalling pathways by growth factors.

The association of SH2 domains with tyrosine phosphorylated sites is also apparently involved in a network of cytoplasmic protein-protein interactions that follow growth factor stimulation. For example, the SH2 domains of GAP bind at least two tyrosine phosphorylated proteins, p62 and p190, that are candidates for regulators and targets of GAP. In addition, the c-Src SH2 domain can apparently interact with its own tyrosine phosphorylated C-terminal tail, and may thereby inhibit Src tyrosine kinase activity. Following Src activation, a number of tyrosine phosphorylated proteins associate with the Src SH2 domain. The biological consequences of these SH2-mediated interactions are under investigation.

H 008 ROLE OF COLONY-STIMULATING FACTOR 1 (CSF-1) IN CELL CYCLE PROGRESSION, Charles J. Sherr[1,2], Hitoshi Matsushime[1,2], and Martine F. Roussel[1], Department of Tumor Cell Biology[1] and the Howard Hughes Medical Institute[2], St. Jude Children's Research Hospital, Memphis, TN 38105

CSF-1 is required throughout the G_1 phase of the macrophage cell cycle to stimulate proliferation and ensure cell survival, but once macrophages enter S-phase, they can complete cell division in the absence of the growth factor. CSF-1 deprivation leads to cell cycle arrest in early G_1, so that if transiently starved macrophages are restimulated, they enter the cell cycle synchronously. If CSF-1 is again withdrawn, the cells fail to enter S phase unless CSF-1 is present throughout the entire G_1 interval. Therefore, CSF-1 must minimally regulate steps at two cell cycle restriction points, temporally corresponding to both early and late G_1.

Stimulation of transiently starved macrophages with CSF-1 activates the intrinsic protein tyrosine kinase activity of the CSF-1 receptor (CSF-1R), thereby initiating a cascade of events that leads to the induction of immediate early response genes. Studies of CSF-1R mutants that are defective in some, but not all, aspects of signal transduction indicate that receptor signals are simultaneously relayed through multiple pathways that act in concert to determine the magnitude and specificity of the biological response. For example, when transduced into NIH/3T3 cells, a CSF-1R mutant containing a Phe for Tyr substitution at codon 809 exhibits nearly wild-type levels of ligand-dependent PTK activity, associates with a phosphatidylinositol 3-kinase, and induces the immediate early response genes, *c-fos* and *junB*. However, cells bearing CSF-1R [Phe-809] are defective in their *c-myc* response and are concomitantly impaired in their ability to be cotransformed by ligand, to form CSF-1 dependent colonies in semisolid medium, and to grow in serum-free medium containing CSF-1. The enforced expression of *c-myc* in these cells restores the wild-type phenotype, indicating that *c-myc* is necessary for CSF-1-induced G_1 progression.

Genetic targets of the CSF-1-induced delayed early response include novel cyclin-like (*CYL*) genes expressed in G_1. *CYL1* is induced in early G_1 and its mRNA and protein levels remain elevated throughout the cell cycle as long as CSF-1 is continuously present. In contrast, *CYL2* is induced later in G_1, but its expression is periodic, being maximal at the G_1/S transition. Induction of *CYL1* and *CYL2* mRNAs by CSF-1 requires new protein synthesis, and the mRNAs and proteins are rapidly degraded (half life <2hr) when CSF-1 is withdrawn. *CYL*-coded proteins (p36) form complexes during G_1 with polypeptides that are immunologically related, but probably nonidentical, to the cell division cycle protein kinase, $p34^{cdc2}$. However, immune complexes containing $p36^{cyl1}$ lack histone H1 kinase activity. Possibly, certain of the $p36^{cyl}$ proteins might negatively regulate the activity of *cdc2* kinases or, alternatively, might target them toward other substrates. Attractive candidates include the retinoblastoma gene product ($p105^{Rb}$), which functions normally to suppress S phase entry and is presumed to be inactivated in late G_1 as a result of its phosphorylation by a *cdc2*-like enzyme. Another member of the *CYL* gene family (*CYL3*) is not expressed in macrophages, but has been detected in other hematopoietic cell lines. In human peripheral blood T lymphocytes, *CYL1* is not expressed; *CYL2* induction appears to be at least partially dependent upon IL-2 receptor activation; and *CYL3* is induced later in G_1 just prior to S-phase entry (J.D. Griffin and F. Ajchenbaum, personal communication). The nucleotide sequences of the murine *CYL* genes are more related to their human cognates than to each other. Together, these results suggest that *CYL* genes respond to different growth factor signals and are likely to regulate distinct cell cycle transitions.

Growth Regulation By RAS and GAP (Joint)

H 009 REGULATION OF RAS p21 AND K-rev1 PROTEINS BY GTPase ACTIVATING PROTEINS (GAP)s, Frank McCormick[1], George Martin[1], Gail Wong[1], Paul Polakis[1], Robin Clark[1], Gideon Bollag[1], Bonnee Rubinfeld[1], Atsuko Yatani[2], and Arthur Brown[2], [1]Cetus Corporation, Emeryville, CA 94608 and [2]Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030.

Ras p21 is regulated by at least three distinct GTPase Activating Proteins (GAP)s, each of which converts active, GTP-bound ras p21 to the inactive, GDP-bound form. p120-GAP and p100-GAP are generated from the same gene by alternative splicing events. These proteins contain regions of homology with src oncogenes (SH2 and SH3 regions) which allow GAP to interact with specific proteins containing phosphotyrosine. PDGF-receptor is the best known of these proteins. The ability of p120-GAP and p100-GAP to interact with ras p21 and tyrosine kinase substrates suggests that GAP connects ras and tyrosine kinase signalling pathways. We have studied the nature of this connection using a cell-free system in which recombinant GAP has a measureable biochemical activity. In this system, GAP uncouples a muscarinic receptor from its G-protein. This effect of GAP is ras dependent. We have found that the SH2/SH3 region of GAP is sufficient for this effect, and that this isolated domain acts in a ras-independent manner. Based on these data, we propose that the ras-binding region of GAP is a regulatory domain that normally prevents GAP's SH2/SH3 region from binding its target. This constraint is relieved by binding ras p21, or, in the cell-free system by removal of the domain. We are currently testing whether ras regulates binding to PDGF-receptor, and other target proteins. One of these targets is p62, a major tyrosine phosphoprotein in many transformed cells. We have cloned this protein, and find it has strong homology to hnRNP and snRNP proteins, suggesting a role in mRNA metabolism.

The product of the gene responsible for neurofibromatosis type 1 (NF1) is also a GAP for ras p21. NF1-GAP has a very high affinity for ras p21: the binding constant for the oncogenic leucine-61 H-ras protein to NF1 is 2 nM. Normal ras p21 also binds tightly to NF1-GAP, and this interaction is stabilized by signalling lipids, such as PIP2 and phosphatidic acid. These compounds inhibit the GTPase effect of NF1-GAP on ras p21, but allow tight binding. A model will be presented in which GAP and NF1 are dual effectors of ras p21 action, that allow ras p21 to co-ordinate two signalling pathways. One of these pathways includes tyrosine kinases (the p120-GAP pathway) and other relates to lipid metabolism (the NF1-GAP pathway).

The biological activity of ras p21 is inhibited by K-rev1, a protein 50% identical to ras p21. The GTPase activity of K-rev1 is regulated by at least one GAP. We have cloned K-rev1 GAP, and examined its biological and biochemical activities. K-rev1 GAP is unrelated to p120 ras GAP or NF1 ras GAP, but has some interesting structural properties, such as sites for phosphorylation by kinase A and p34. We will discuss the possible relationship between K-rev1 GAP action and ras p21.

H 010 STUDY ON THE *Krev*-1 TRANSFORMATION SUPPRESSOR GENE, Makoto Noda[1], Hitoshi Kitayama[2], Naoki Nishino[2], Tomoko Matsuzaki[1], and Yoji Ikawa[2, 3], [1]Cancer Institute, Japanese Foundation for Cancer Research, Kami-Ikebukuro, Toshima-ku, Tokyo 170, [2]Tsukuba Life Science Center, The Institute of Physical and Chemical Research, Koyadai, Tsukuba, Ibaraki 305, [3]Tokyo Medical and Dental University School of Medicine, Yushima, Bunkyo-ku, Tokyo 113, Japan.

Krev-1 cDNA was originally recovered from a "flat" revertant (R16) derived from Kirsten sarcoma virus-transformed NIH3T3 cells (DT line) after transfection of a pcD2-based human fibroblast cDNA expression library followed by enrichment for cells with increased attachment to the plastic dish. The *Krev*-1-encoded protein was found to be identical to *Rap* 1a and *smg* p21, independently discovered by other groups through different approaches, and it shares around 50% amino acid identity with Ras proteins. Besides the conserved motifs commonly found in G proteins, Krev-1 and Ras share an identical amino acid sequence in the region know as the effector-binding domain. The carboxy terminal region of the *Krev*-1 protein contains two characteristic structures playing roles in membrane association: lysine-repeats and a CAAL box. The latter serves as the signal for geranylgeranylation on the cysteine residue. The serine residue adjacent to the CAAL box has been shown to serve as a target for A-kinase. Two types of proteins modulating the biochemical activities of the *Krev*-1 protein (i. e. GDP/GTP-binding and GTPase) have been found: GTPase activating proteins (rap1 GAPs) and GDP-dissociation stimulator (*smg* GDS). The *smg* GDS has an additional activity to remove *smg* p21 from the membrane and this process is accelerated upon the serine-phosphorylation of *smg* p21 by A-kinase. The *Krev*-1 gene and/or its close homologue (*rap1B*) is expressed ubiquitously in various tissues and especially abundant in neurons, platelets and neutrophils. Association of the *Krev*-1 protein with the Golgi apparatus in fibroblasts has been demonstrated.

When *Krev*-1 expression plasmid is transfected into DT cells, a few percent of the transfectant colonies show flat morphology, and only a subtle morphological changes could be found in the rest of the transfectants. A part of the reason for this low efficiency of revertant-induction may be explained by the model in which the Krev-1 protein is switched-on in response to an unknown upstream signal. Although certain point mutations in the *Krev*-1 gene, that are expected to impair the intrinsic GTPase activity of its product ("activating" mutation), increased the efficiency of revertant-induction up to 5 fold, more than 80% of the transfectants still remain transformed. These findings may suggest the existence of additional regulatory mechanisms for the Krev-1 protein, besides the GDP/GTP-exchange reaction, and/or the existence of clonal heterogeneity among the recipient DT cells in the levels of molecules essential for the Krev-1 protein in exerting its biological effects. To understand the molecular basis underlying the apparently opposing biological activities between Ras and Krev-1, we previously analyzed the transforming and the revertant-inducing activities of a series of chimeric H-*ras*-*Krev*-1 genes, and found that small clusters of divergent amino acid residues surrounding the putative effector-binding domain are probably responsible for the difference in the activities. Recently, we have examined the *Krev*-1 as well as H-*ras* genes carrying amino acid substitutions at these divergent residues, and found that substitutions of two amino acid residues in H-Ras protein to Krev-1 type amino acids (D30E and E31K) are sufficient to convert the oncogenic H-Ras protein to a transformation suppressor. To examine whether alterations of the *Krev*-1 gene is involved in the process of human carcinogenesis, we are trying to: 1) develop a retrovirus-based *Krev*-1 expression system to test its effects on various types of tumor cells, and 2) to determine the structural of the genomic *Krev*-1 gene to facilitate the survey of possible gene alterations in tumors samples. In the first line of experiments, we have found that the "activated" *Krev*-1 gene exibits growth suppressing or tumor suppressing activity on certain cell lines derived from fibrosarcoma and colorectal carcinoma. In the second, we have learned so far that the *Krev*-1 gene is split by at least 6 introns (3 in the coding region), and there is at least one pseudogene exists in the human genome.

Cytoplasmic Tyrosine Kinases

H 011 SIGNAL TRANSDUCTION EVENTS INVOLVED IN NEURONAL DIFFERENTIATION OF PC12 CELLS, Sheila Thomas[1], Michael DeMarco[1], Gabriella D'Arcangelo[2], Simon Halegoua[2], and Joan S. Brugge[1]. [1]Howard Hughes Medical Institute, Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania, [2]Department of Neurobiology and Behavior, State University of New York at Stony Brook, Stony Brook, New York

PC12 cells serve as a model system for investigating the mechanisms involved in neuronal growth factor induced differentiation. Nerve growth factor (NGF), fibroblast growth factor (FGF), and oncogenic forms of Src and Ras induce PC12 cells to differentiate into cells resembling sympathetic neurons. We have previously shown that microinjection of monoclonal antibodies to either Ras or Src prevent NGF and FGF induction of neuronal differentiation*, suggesting that the cellular protein tyrosine kinase, pp60$^{c\text{-}src}$, and at least one form of p21^{ras} are essential for NGF- and FGF-induced differentiation.

To further investigate the involvement of Src and related protein tyrosine kinases, we have examined the catalytic activity of these enzymes following growth factor stimulation using immunocomplex kinase assays. In addition, we are attempting to dissect the intracellular pathways regulated by Src and Ras, as well as other cytoplasmic signaling molecules by using activated or dominant-interfering variants of these proteins.

*Hagag, N., S. Halegoua, M. Viola. Nature 319:680-682.

Kremer, N. G. D'Arcangelo, S.M. Thomas, M. DeMarco, J.S. Brugge, S. Halegoua. J. Cell Biol., in press.

H 012 THE ROLE OF SH2 DOMAINS OF SRC AND CRK PROTEINS, Hidesaburo Hanafusa[1], Raymond Birge[1], Eduardo Fajardo[1], Charles Reichman[1], Bruce Mayer[1], Hisataka Sabe[1], Beatrice Knudsen[1], Siyun Fang[1], Masato Okada[2], Shigeyuki Nada[2], and Hachiro Nakagawa[2], [1]Laboratory of Molecular Oncology, The Rockefeller University, New York, NY 10021, [2]Division of Protein Metabolism, Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan.

SH2 and SH3 domains have been identified in a number of proteins involved in early processes of signal transduction. Since the association of the SH2 sequences with cellular proteins is dependent on tyrosine phosphorylation, it has been proposed that this type of protein-protein interaction is important for growth promoting signals. Furthermore, the protein encoded by the viral crk oncogene consisting primarily of SH2 and SH3 sequences induces increases in the level of phosphotyrosine (ptyr)-containing proteins in transformed cells. This indicates that the Crk SH2 and SH3 domains can modulate tyrosine phosphorylation of cellular proteins in trans, since Crk does not contain intrinsic tyrosine kinase activity. Analysis of this system showed that the SH2 rather than SH3 domain contributes to the binding.

There is a specificity in the interaction between SH2 sequences and ptyr-containing proteins: not all combinations of these two types of proteins result in stable association. In order to characterize the specificity of interaction, we have developed an in vitro microtiter assay in which binding of phosphorylated EGF receptor with v- and c-Crk proteins was measured. We found that their binding takes place with an apparent Kd of approximately 1 x 10^{-9} M. The binding can be quantitatively competed by GST-fusion proteins containing Crk as well as GAP SH2 but less by those containing Abl and Src SH2 regions. We also found that the binding of Crk protein to phosphorylated EGF receptor resulted in a significant protection against dephosphorylation by cellular phosphatase activity. This may be a major mechanism contributing to the increased level of phosphotyrosine in Crk-transformed cells.

Nonreceptor-type protein tyrosine kinases including the Src-family proteins are unique in having both SH2 domain and phosphorylated tyrosine residues. Several pieces of evidence suggest that the carboxyl tail of the Src protein containing Tyr527 interacts with its own SH2 domain located in the amino terminal half. Recently, CSK (C-terminal Src Kinase) was isolated as a candidate protein kinase that specifically phosphorylates Tyr527. Purification and cloning of CSK demonstrated that it also has SH2 and SH3 domains in its amino terminus. It is conceivable that these sequences play a role in its interaction with c-Src protein. However, we have not detected the association of these two proteins in vivo and in vitro.

H 013 PROTEIN TYROSINE PHOSPHORYLATION IN GROWTH CONTROL AND THE CELL CYCLE Tony Hunter, Rick Lindberg, David Middlemas, Peter van der Geer, Sharon Tracy and John Pines The Salk Institute, La Jolla, California 92037

We have been studying 2 novel receptor-like protein-tyrosine kinases (PTKs). One of these, Eck, is mainly expressed in tissues containing growing epithelial cells, and is expressed on the basolateral surface of epithelial cell layers. Eck is a member of a small family of receptor PTKs, which includes Eph, Elk and Eek. Analysis of the 120 kDa Eck protein in immunoprecipitates from epithelial cells shows that Eck is a PTK. In an attempt to identify the Eck ligand, we have expressed Eck in NIH3T3 mouse fibroblasts and in 32D mouse myeloid cells, and we are currently searching for tissue extracts or cell conditioned media that can stimulate Eck Tyr phosphorylation in these cell lines. The second PTK, TrkB, isolated from a rat cerebellar library 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. Analysis of *trk*B cDNAs indicates that there are mRNAs encoding 2 different short forms of TrkB, which are both truncated just downstream of the transmembrane domain, and have short distinct C-termini. Using antisera to TrkB we have identified the full length protein as a 140 kDa glycoprotein. Based on the observation that NGF binds to and stimulates Trk PTK activity, we have shown that TrkB PTK activity is stimulated by NT-3 and BDNF, and are searching for substrates for TrkB. We have identified Tyr706 in the kinase insert region of the CSF-1 receptor as an autophosphorylation site, and shown by mutagenesis that the phosphorylation of Tyr706 plays a role in induction of immediate early genes by CSF-1. We have identified Tyr721 as the phosphorylation site responsible for the binding of PI-3 kinase to the activated CSF-1 receptor. We are examining a receptor-like P.Tyr-phosphatase, PTPα, that is a phosphoprotein expressed in fibroblasts. We are currently testing whether its phosphorylation state and PTPase activity change in response to mitogenic stimulation.

Cyclins are a family of proteins implicated in the induction of mitosis, which are marked by their accumulation in interphase followed by dramatic destruction at mitosis in each cell cycle. We have isolated human A- and B1-type cyclin cDNA clones, and raised antisera to recombinant cyclins. We have found that the level of both cyclins increases during G2 to reach a peak at metaphase, when both are destroyed. Cyclin B1 forms a complex with $p34^{cdc2}$ in late S and G2, but this complex is not activated as a histone H1 kinase until $p34^{cdc2}$ is dephosphorylated at the end of G2. In contrast, from S phase onwards cyclin A associates with $p34^{cdc2}$ and with a protein of ~33 kDa that is similar to, but distinct from $p34^{cdc2}$. Using PCR we have isolated five $p34^{cdc2}$-related cDNAs encoding ~33-35 kDa proteins containing the PSTAIR motif and the hallmarks of serine kinases, which are distinct from $p34^{cdc2}$. We have evidence that one of these encodes p33 and interacts cyclin A. The H1 kinase activity of the cyclin A/p33 complex gradually increases up to M phase, in parallel with the amount of cyclin A. Cyclin B1 accumulates in the cytoplasm of G2 cells and moves into the nucleus just as cells enter mitosis, before nuclear lamina breakdown. In prophase cyclin B1 localizes to the mitotic asters, and in metaphase cyclin B1 localizes to the spindle poles and microtubules. In contrast to cyclin B, cyclin A is predominantly a nuclear protein, and cell fractionation studies suggest that p33 is almost exclusively associated with the nuclear form of cyclin A. We are analyzing cyclin A/B1 chimeras to determine what dictates the localization of the two cyclins. We propose that the cyclin $B1/p34^{cdc2}$ complex is the primary mitotic kinase and that its function is regulated by nuclear translocation, whereas the cyclin A/p33 complex acts earlier in the cell cycle and could be involved in the process of DNA replication. The differential localization of cyclins A and B1 may allow the 2 cyclins to play different roles in the cell cycle in association with their distinct cdc2-related protein kinase subunits by targetting them to different substrates.

H 014 ROLE OF THE *lck* TYROSINE PROTEIN KINASE IN T CELL ACTIVATION, Bartholomew M. Sefton, Tamara Hurley, Kunxin Luo, and Robert Hyman, The Salk Institute, San Diego, California

Stimulation of T lymphocytes through the antigen receptor induces increased tyrosine protein phosphorylation, elevated free cytoplasmic calcium, and activation of protein kinase C. T lymphocytes that do not express the cell surface tyrosine protein phosphatase CD45 show none of these changes after stimulation. This suggests that tyrosine dephosphorylation must precede increased phosphorylation of proteins on tyrosine, elevation of cytoplasmic calcium, and activation of protein kinase C during T cell activation. Of interest therefore is the identity of the substrate or substrates of CD45.

The *lck* tyrosine protein kinase is one apparent substrate of CD45 in the SAKR murine T cell line. It is phosphorylated to a 3 fold greater extent at a site of inhibitory phosphorylation in a variant of the SAKR T cell line that does not express CD45. CD45 therefore has the properties of a natural activator of the *lck* kinase.

We are interested in the generality and specificity of the interaction of CD45 and the *lck* kinase. T lymphocytes express a number of tyrosine protein kinases besides $p56^{lck}$. One of these is the *fyn* protein, a 59,000 dalton tyrosine protein kinase that is a close relative of $p56^{lck}$. Because it can be recovered in association with the T cell receptor if cells are lysed under gentle conditions, $p59^{fyn}$ may play a role in T cell activation. We have examined the phosphorylation of the *lck* and *fyn* protein kinases in three lines of CD45-negative T cells. The *lck* kinase was phosphorylated to an increased extent at the inhibitory site in all three cell lines. In contrast, the phosphorylation of the *fyn* kinase was unaltered in CD45-negative cells. This suggests that CD45 exhibits considerable polypeptide substrate specificity. Additionally, these results are consistent with the idea that it is the dephosphorylation of the *lck* kinase by CD45 that is critical in signal transduction during T cell activation.

Second Messenger Systems

H 015 THE EGF RECEPTOR AND TYROSINE PHOSPHORYLATION OF PHOSPHOLIPASE C-γ1, Graham Carpenter, Departments of Biochemistry and Medicine, Vanderbilt University School of Medicine, Nashville, TN 37232-0146

Distinct and interrelated aspects of the EGF receptor and phospholipase C-γ1 (PLC-γ1), a major substrate for the tyrosine kinase activity of the EGF receptor and several other receptors, will be discussed. Studies of multiple autophosphorylation site mutations of the EGF receptor will be presented in terms of their effects on endocytosis and the phosphorylation of exogenous substrates such as PLC-γ1. These studies indicate that mutagenesis of more than two autophosphorylation sites restricts receptor function, but mutagenesis of two or one sites does not. Additional studies of EGF receptor dimerization indicate that dimers are present in internal cell compartments as well as on the cell surface. Intracellular dimers are, as a percentage of the total intracellular receptor population, as prevalent as dimers on the cell surface.

Studies of tyrosine phosphorylated PLC-γ1 will center on the contribution of tyrosine phosphorylation to the mechanism of action of PLC-γ1, as assessed by in vitro assays of PLC-γ1 activity. Factors, such as the presence of the EGF receptor or phosphatidic acid, which influence the activity of PLC-γ1 will be elaborated. Particular emphasis is to be placed on the catalytic activity of PLC-γ1 and its regulation. Kinetic data suggests that tyrosine phosphorylation enhances the association of PLC-γ1 with substrate micelles. Lastly, the localization of PLC-γ1 in tissues will be reported. This will include data on human mammary carcinomas, benign hyperplastic disorders (e.g. psoriasis), and normal tissue. PLC-γ1 seems to be overexpressed in a large percentage of the mammary carcinomas.

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Oncogenes and Differentiation I

H 016 THE MANY FACES OF FOS. Tom Curran, Department of Molecular Oncology & Virology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110

c-*fos* and c-*jun* are members of the set of cellular immediate-early genes. Their protein products are components of the mammalian transcription factor Activator Protein-1 (AP-1). They are thought to function as nuclear "third-messenger" molecules in coupling cell surface signals to changes in cellular phenotype by regulating expression of selected target genes. The situation is complicated by the existence of several Fos- and Jun-related genes and selected ATF/CREB family members that can form leucine zipper dimers with Fos and Jun. Different dimers exhibit differential specificity and affinity for AP-1 and CRE sites. Because of this complexity, it is likely that cellular mechanisms exist that are capable of selecting among different homo- and heterodimeric complexes.

Two such potential mechanisms have been identified. Fos and Jun are phosphorylated efficiently by several protein kinases including, $p34^{cdc2}$, casein kinase II, protein kinase C, protein kinase A and DNA-dependent protein kinase. However, phosphorylation depends on whether the proteins are in the monomeric (Fos), homodimeric (Jun) or heterodimeric (Fos-Jun) state. In addition, the presence of the AP-1 site can either inhibit or stimulate phosphorylation depending on the kinase used.

The DNA-binding activity of Fos and Jun is also influenced by an unusual reduction/oxidation (redox) mechanism. The proteins are readily modified to an unusual oxidation state that does not involve disulfide bond formation, that inhibits DNA binding. Stimulation of DNA-binding activity can be achieved either by using elevated levels of reducing agents or by a cellular protein that we have identified. It is likely that these and other mechanisms operate in concert to regulate the specificity and activity of Fos, Jun and related immediate-early transcription factors.

Regulation of Gene Expression (Joint)

H 017 RETINOID RECEPTORS IN DEVELOPMENT AND DISEASE, Ronald M. Evans[1], Akira Kakizuka, Steve Kliewer David Mangelsdorf, and Kazuhiko Umesono[1], Howard Hughes Medical Institute[1], The Salk Institute, La Jolla, CA 92037.

The cellular responses to RA are mediated by two families of transcription factors, which include the RA receptors (RARs) and the retinoid X receptors (RXRs). Although both RAR and RXR respond specifically to RA, they differe substantially from one another in primary structure and ligand specificity. A major question raised by the discovery of two retinoid-responsive systems is whether their functions are independent, interactive, or redundant. One approach to answer this question is to determine whether they share common or distinct downstream target genes. In regard to target sequences we have recently described properties of direct repeats (DRs) of the half-site AGGTCA as hormone response elements. According to our results, spacing of the half-site by 3, 4, or 5 nucleotides determines specificty of response for vitamin D3, thyroid hormone and retinoic acid receptors, respectively. This so-called "3-4-5" rule suggests a simple physiologic code exists in which half-site spacing plays a critical role in achieving selective hormonal response. As part of these studies, we have also identified that the RXR, but not the RAR, is able to activate through a direct repeat spaced by one nucleotide. In contrast, both RAR and RXR are able to commonly activate through a DR with a spacing of 5. Evidence that RXR heterodimers modulate the RA response will be presented.

Finally we will discuss the isolation and characterization of a fusion product produced as a consequence of a t(15;17) translocation characteristic of human acute promyelocytic leukemia. This translocation which occurs in the retinoic acid receptor gene generates a unique mRNA which encodes a fusion protein between the retinoic acid receptor alpha (RARα) and a myeloid gene product called PML. Structural analysis reveals that the PML protein is a member of newly recognized protein family that includes a variety of putative transcription factors as well as the recombination-activating gene product (RAG-1). The abberant PML-RAR fusion product, while typically retinoic acid responsive, displays both cell type and promoter specific differences from the wild type RARα. Because patients with APL be induced into remission with high dose RA therapy, we propose that the non-liganded PML-RAR is a new class of dominant negative oncogene product.

H 018 ACTIVATION AND ANTIREPRESSION OF TRANSCRIPTION BY PROMOTER- AND ENHANCER-BINDING FACTORS, Paul J. Laybourn, Rohinton T. Kamakaka, Glenn E. Croston, and James T. Kadonaga, University of California, San Diego.

Transcription by RNA polymerase II involves the basal transcriptional machinery, sequence-specific DNA binding factors that interact with promoter and enhancer elements, and the template DNA, which is packaged into chromatin. In addition, it appears that there may be another class of factors, which have been named coactivators, mediators, or adapters, that are required for activation of transcription by the promoter- and enhancer-binding factors. The basal transcriptional apparatus comprises RNA polymerase II as well as several auxiliary factors, which are generally referred to as the general factors. Several of the general factors, including the TATA-box binding polypeptide of TFIID, TFIIA, TFIIB, TFIIE, and TFIIF, have been purified and cloned from various organisms, but the exact number of general factors that are required for basal transcription has not yet been elucidated. Furthermore, it is not known whether all promoters require the same set of general factors. The first portion of the lecture will describe our recent studies on the purification, characterization, and cloning of the general transcription factors from Drosophila. The remainder of the talk will be devoted to the antirepression hypothesis and the use of chromatin templates for transcription in vitro. Briefly, it can be imagined that promoter- and enhancer-binding factors may activate transcription by either or both of the following two mechanisms. First, the sequence-specific factors may facilitate the *inherent transcription reaction -- we refer to this as "true activation."* Alternatively, the promoter- and enhancer-binding factors may counteract a general repression of basal transcription by a nonspecific DNA binding entity (*i.e.*, chromatin) -- we have designated this effect as "antirepression." In our earlier studies on transcriptional activation in vitro, we had found that Sp1 (which contains a glutamine-rich activation region) and the GAL4-VP16 fusion protein (which contains an acidic activation region) were capable of both true activation and antirepression. The antirepression effect was unexpected, however, because chromatin assembly did not occur under the reaction conditions. We thus purified the DNA binding repressor and identified the protein as histone H1. A number of studies, both in vivo and in vitro, have suggested that H1 is involved in transcriptional repression. We have extended these studies with chromatin templates, and it presently appears that promoter- and enhancer-binding factors function both for true activation and antirepression and that under certain circumstances, there is a competition for promoter binding between the sequence-specific factors and histone H1 that leads to either gene activation or repression.

H 019 STRUCTURE AND FUNCTIONAL INTERACTIONS OF GENERAL INITIATION FACTORS, REGULATORY FACTORS AND COFACTORS, Robert G. Roeder, Ananda Roy, Micheal Meisterernst, Philippe Pognonec, Yan Luo, and Hiroshi Fujii, Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10021.

Continued studies of the purification and characterization of general factors, which are ubitquitous and commonly required by most class II genes, have implicated factors IIA, IIB, IID, IIE, IIF, IIG, and II-I in transcription initiation from minimal (core) promoters by RNA polymerase II. Amongst these factors, TFIIA and TFII-I are functionally interchangeable and form alternate preinitiation complexes on the adenovirus ML promoter, which suggests the possibility of selective responses to different regulatory factors. Unlike TFIIA, TFII-I binds stably to a number of initiator elements, but, like TFIIA, interacts cooperatively with TFIID. TFII-I also has been shown to interact cooperatively with the upstream activator USF (a helix-loop-helix protein), suggesting a novel mechanism for communication between regulatory factors and the general transcriptional machinery. The cloning of TFII-I has revealed structural relationships with helix-loop-helix proteins.

Studies of the AdML and HIV promoters have identified a novel cofactor (USA) that is required for physiological levels of promoter induction in purified systems (with general factors) by USF (AdML), Sp1 (HIV) and NFkB (HIV). The action of USA involves both a large net increase in promoter activity in conjunction with the activator and a repression of activator-independent activity. Fractionation and mechanistic studies indicate the involvement of both a negative cofactor (NC1) which competes with TFIIA for binding to TFIID, leading to basal repression , and a positive cofactor (PC1) which, with the activator, reverses the action of NC1 and effects a large positive promoter response. These results, along with the identification of other negative and positive factors interacting with TFIID, suggest novel promoter regulatory mechanisms that differ from previous models involving simple adaptors. Although the action of USF appears general, the possibility of activator-specific components has not been ruled out.

Related studies have revealed that the ubiquitous Oct1 and the lymphoid-restricted Oct2 are indistinguishable with respect to their intrinsic abilities to activate immunoglobulin (Ig), H2B, or snRNA promoters but that they interact with additional factors in a promoter-specific way to enhance transcription by the common factors. The isolation and characterization of a novel B cell-specific accessory factor (BAF) that specifically and markedly enhances Ig promoter activity via Oct1 or Oct2 suggests the existence of a new class of tissue specific cofactors/coactivators that may play a major role in the determination of promoter specificity and activity in vivo.

Oncogenes and Differentiation II

H 020 ONCOGENE IMPACT ON HEMATOPOIETIC CELLS, Jerry M Adams, The Walter and Eliza Hall Institute of Medical Research, Post Office, Royal Melbourne Hospital, Parkville, Victoria 3050, Australia.

To explore how different oncogenes impinge on hematopoietic differentiation and contribute to neoplasia, we have constructed transgenic mice in which expression is driven by an immunoglobulin enhancer (Eμ), which functions throughout the B lymphocyte lineage and occasionally in T cells and certain myeloid cells. The resulting models of tumor development involve pre-B and B lymphomas (*myc*, N-*myc*, v-*abl*), plasmacytomas (v-*abl*), T lymphomas (N-*ras*, v-*abl*) and macrophage tumors (N-*ras*). Preneoplastic effects have varied markedly. Whereas N-*ras* and v-*abl* appear to have no detectable effect on lympho-hematopoietic development, *myc* and N-*myc* markedly increase the number of cycling pre-B cells. In contrast, the *bcl*-2 gene is a prototypic oncogene that promotes cell survival rather than proliferation. Within the B cell lineage, it evokes a great excess of non-cycling B cells which survive factor deprivation *in vitro*, and T cell longevity is also enhanced.

In all the transgenic models, tumors arise stoichastically due to the need for spontaneous alterations in genes that collaborate with the transgene. For example, *ras* is sometimes mutated in Eμ-*myc* lymphomas, and lymphomagenesis was accelerated in mice bearing both a *myc* and N-*ras* transgene. Striking cooperativity also held for *myc* and *bcl*-2; surprisingly, all the tumors that arose represented a primitive hematopoietic cell type, perhaps a lymphoid stem cell.

To identify other genes that can collaborate with *myc*, we have exploited insertional mutagenesis. Since the tumorigenic action of retroviruses that lack an oncogene, such as Moloney murine leukemia virus, mainly reflects chance insertion near cellular oncogenes, the provirus tags the relevant gene. Neonatally infected Eμ-*myc* mice rapidly developed pre-B lymphomas, and half the accelerated lymphomas bore proviral inserts near a novel gene denoted *bmi*-1. It encodes a new type of Zn finger and most likely represents a transcription factor that collaborates with *myc*.

Retroviral vectors facilitate gene delivery to non-lymphoid hematopoietic cells. The finding that a myeloid leukemia contained endogenous retroviral-like (IAP) inserts near a homeobox gene (Hox-2.4) and the gene for the myeloid growth factor IL-3 suggested that both might contribute to leukemogenesis. Indeed a retrovirus bearing both genes provoked rapid myeloid leukemia in normal bone marrow cells. The Hox-2.4 gene appears to promote immortalization of myeloid cells by preventing terminal differentiation.

H 021 REGULATION OF THE HUMAN β-GLOBIN GENE, Frank Grosveld, Laboratory of Gene Structure and Expression, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

The human β-globin gene family contains five functional genes. These are arranged in the same order as they are expressed during development, i.e. 5'-ϵ-γ_G-γ_A-δ-β-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 Locus Control Region (LCR). This LCR 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 (GATA-1 and NF-E2) and one non-erythroid protein to be essential for the function of this region. Addition of the LCR 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.

H 022 THE EVI-1 ZINC FINGER TRANSCRIPTIONAL FACTOR AND MYELOID LEUKEMOGENESIS, James N. Ihle, Kazuhiro Morishita, Evan Parganas, Takayasu Matsugi, Tetsunori Funabiki, Rudd Delwel and Brent Kreider, Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee 38101.

Insertional activation of the *Evi-1* gene is one of the most common transforming events associated with murine, retrovirus-induced, myeloid leukemias. Activation of expression occurs due to insertions in the 5' region of the gene or can also occur by retroviral insertions 90 kb 5' of the gene in a 15 kb region that was initially identified as the CB-1/*fim*-3 common site of integration. The *Evi-1* gene product contains two domains of zinc fingers. The consensus binding sequence for both the seven finger amino terminal domain and the carboxyl three finger domain have been determined. The properties of the myeloid cell lines in which the gene is activated suggest that transformation by the *Evi-1* gene primarily involves an alteration in the ability to terminally differentiate. Consistent with this, introduction of the *Evi-1* gene into myeloid cells that have the ability to terminally differentiate to granulocytes in the presence of G-CSF eliminates this property. The mechanisms involved are not known, but may result from competition of the *Evi-1* gene product with transcriptional factors that are required for differentiation. Activation of expression of the *Evi-1* gene occurs in approximately 7% of the cases of human AML. In three cases that have been characterized, the translocations or inversions that activate the gene occurred 200 kb or 15 kb 5' of the gene or 150 kb 3' of the start of the gene. The phenotypic properties of the human AMLs in which the *Evi-1* gene is activated support the hypothesis that aberrant expression interferes with the ability of the cells to terminally differentiate.

Growth and Development

H 023 THE DROSOPHILA *ABL* TYROSINE KINASE: ELUCIDATING ITS DEVELOPMENTAL REQUIREMENTS AND IDENTIFYING INTERACTING MOLECULES, F. Michael Hoffmann, Frank Gertler, Randy Bennett, Kevin Hill, Mark Henkemeyer, and Michael Clark, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison WI 53706

In Drosophila, *abl* protein expression is most easily detected in early embryos, neural axons, and muscles although lower levels of expression are detected in a variety of tissues throughout most stages of development. Animals lacking functional *abl* survive until advanced pupal or adult stages; these mutants exhibit mild pleiotropic defects and are short lived, but otherwise appear relatively normal. We have employed genetic strategies to isolate second-site mutations which either exacerbate or alleviate the *abl* mutant phenotype. *abl* mutant animals which carry heterozygous mutations in one of five genes identified in this screen die during embryogenesis and exhibit defects in the axons of the central nervous system; these defects are fully rescued by the presence of an *abl* transgene. Screens of 11,000 mutagenized chromosomes have recovered multiple alleles in three of the five enhancer loci, indicating that only a few genes are targeted by this genetic strategy.Two of these interacting genes, *disabled* and *prospero* have been cloned and sequenced. *disabled* is expressed in the same tissues as *abl* and shows overlapping subcellular localization. Perhaps most importantly, we have shown that the *disabled* product contains phosphotyrosine in S2 cells which have been cultured in the presence of vanadate. Thus, the *disabled* gene product can be a substrate for tyrosine kinases in vivo. The genetic interactions indicate that one of the kinases that may phosphorylate the *disabled* protein is the *abl* tyrosine kinase. Unlike *disabled*, *prospero* is expressed earlier in neural development than *abl* and shows only additive phenotypic interactions with mutations in either *abl* or *disabled*. We believe its genetic interactions with *abl* are indirect. We have also recovered three mutations in another gene, *enabled*, which suppress the defects observed in both *abl* and *disabled* mutants, but have no effect on *prospero* mutants. Perhaps due to the transient nature of tyrosine phosphorylation and/or its role in modulating transient protein-protein interactions, eliminating the *abl* tyrosine kinase from Drosophila (and also from mice) has minor effects that can be largely compensated for by normal homeostatic mechanisms in the cell. However, elimination of the tyrosine kinase greatly sensitizes the animal to further genetic insults.

Late Abstract

PHOSPHATIDYLINOSITOL 3-KINASE, Lewis Cantley, Dept. of Physiology, Tufts University School of Medicine, Boston MA 02111.

Phosphatidylinositol 3-kinase (PtdIns 3-kinase) was discovered because of its tight association with activated protein tyrosine kinases. The enzyme has been purified to homogeneity and shown to be a heterodimer of 110 KD and 85 KD subunits. The 85 KD subunit contains regions of homology with the non-catalytic domain of $pp60^{src}$ (an SH3 domain and two SH2 domains). It also contains a region of homology with the carboxy-terminal domain of the bcr gene product. A multigene family of p85 proteins exist. In rat liver two alternative splice forms of p85A are found with distinct bcr domains but identical SH2 domains. PtdIns 3-kinase associates with both growth factor receptor type and cytosolic protein-tyrosine kinases. The mechanism of association with these two types of tyrosine kinases are distinct. Autophosphorylation of the cytosolic domains of certain growth factor receptor tyrosine kinases creates a binding site for the SH2 domains of p85 resulting in recruitment of PtdIns 3-kinase from the cytosol to the inner surface of the plasma membrane. Based on similarity with the region of the polyoma middle t gene product that associates with PtdIns 3-kinase (Phospho-Tyr-315), two related 6 amino acid consensus sequences for the tyrosine phosphorylation sites that associate with p85 have been determined. Several growth factor receptors contain one or more of these sequences. The major substrate of the insulin receptor (IRS-1) has multiple copies of both types of sequences and associates with PtdIns 3-kinase in insulin-stimulated cells. Synthetic phosphopeptides based on the region around middle t Tyr-315 block association of PtdIns 3-kinase with baculovirus-expressed middle $t/pp60^{c\text{-}src}$ complexes *in vitro* confirming that this region is involved in binding. Finally, a manganese-dependent protein-serine kinase copurifies with PtdIns 3-kinase and immunoprecipitates using antibodies against recombinant p85. This enzyme phosphorylates both p85 and p110 and will phosphorylate casein but not histone. It probably accounts for the serine phosphorylation of p85 *in vivo*. A model for regulation of PtdIns 3-kinase activity will be presented.

Cell Cycle

H 100 DIFFERENTIAL EFFECTS OF IMMUNOSUPPRESSIVE DRUGS ON GLIOBLASTOMA CELLS, Chaya Brodie, Joseph Lucas, Agota Szepesi, Paul Seligman and Erwin W. Gelfand, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206

Cyclosporin A (CsA) and FK506 are immunosuppressive agents that have been used effectively in preventing rejection of organ transplants. CsA binds to a cytoplasmic receptor, cyclophilin, while FK506 binds to a distinct, 12 kDa cytoplasmic receptor, FK506 binding protein (FKBP-12). Rapamycin, structurally related to FK506, has been shown to bind and also to inhibit the isomerase activity of FKBP-12. CsA and FK506 have significant neurological clinical side effects including tremor, ataxia, seizures and coma and the presence of both cyclophilin and FKBP-12 has been demonstrated in the brain. We have examined the effects of the immunosuppressive drugs, CsA, FK506 and rapamycin on growth and cell cycle events of the glioblastoma cell line, T98G, a cell line which exhibits a number of properties associated with growth control. Both CsA and rapamycin inhibited cell proliferation in a dose-dependent manner while surprisingly FK506 was without any detectable effect. CsA inhibited the growth of cells by arresting them in G_1/S phase while rapamycin increased the accumulation of cells in the G_1 phase. FK506 did not interfere with cell cycle progression but antagonized the effect of rapamycin when these drugs were added together. Treatment of highly synchronized cells (released from G_0 state) with either rapamycin or CsA resulted in an inhibition of cell proliferation which was paralleled by an inhibition of cdc2 protein as measured by Western blot. The antiproliferative effects of CsA and rapamycin were accompanied by a different morphological appearance of the cells. Thus cells treated with CsA exhibited a round-shape whereas rapamycin-treated cells were elongated with a larger cell body. The differential inhibitory effects of all three drugs on T lymphocytes and the absence of an FK506 effect on the T98G cell line now provide contrasting systems for studying the effects of these drugs at the cell cycle level. The T98G cell line can be also used as a model system to evaluate the neurological effects of these immunosuppressive drugs. In addition, the inhibitory effects of CsA and rapamycin may have potential importance in treatment of human brain tumors which are refractory to conventional chemotherapy.

H 101 RAPID INDUCTION OF COMPETENCE FORMATION IS PDGF-ISOFORM SPECIFIC, Steven R. Coats, James E. Olson and W.J. Pledger, Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232. Platelet-derived growth factor (PDGF) stimulates the expression of a number of genes associated with entry of quiescent Balb/c-3T3 fibroblasts into the cell cycle. We determined that two of these genes, c-myc and c-fos, are induced equivalently in medium supplemented with platelet-poor plasma (PPP) and either PDGF-BB or PDGF-AA. The rate at which fibroblasts entered S phase was also similar in PDGF-BB and AA-treated cells as was the expression of the late G_1 gene, thymidine kinase (TK). However, PDGF-AA must be present for a period of 16 h to stimulate the proliferation of 90% of the cells, whereas PDGF-BB was required for only 4 h. Exposure of cells to PDGF-AA for 4 h, a time during which maximum expression of c-fos and c-myc occurred, only induced 20% of the cells in a quiescent population to enter the cell cycle. Therefore, PDGF-AA-mediated expression of the immediate early genes c-fos and c-myc may be necessary but is not sufficient to rapidly stimulate density-arrested Balb/c-3T3 fibroblasts into the competent state. Thus, these data suggest that PDGF-AA and PDGF-BB initiate traverse of the cell cycle by distinct mechanisms.

H 102 IN VITRO ACTIVATION OF p34-CDC28 BY A G1 CYCLIN, Ray Deshaies and Marc Kirschner, Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0448.

The G1-S transition is the principal point in the cell cycle where eukaryotic cells decide whether to continue growth and cell dividion, or instead to become quiescent or embark upon a program of differentiation. The G1-S transition in the cell cycle of *Saccharomyces cerevisiae* is dependent upon both G1 cyclins (CLNs) and the p34cdc2 homolog, Cdc28p. One intriguing idea is that activation of Cdc28p protein kinase activity, driven by the CLN proteins, serves to couple cell growth with initiation of the cell division cycle. Experimental support for this notion derives from the observation that cells deleted for either one of two CLN genes (*CLN2 or 3*) are larger than normal and exhibit a reduced rate of passage through G1, whereas cells bearing a hyperactive allele of either gene initiate the division cycle at a reduced cell size and respond poorly to negative regulatory cues that normally restrain cells in G1 phase[1-3]. Identification of the biochemical reactions culminating in the activation of Cdc28p protein kinase should provide clues to the strategies that eukaryotic cells employ to couple cell size with cell division. To address the biochemical pathways that promote the G1-S transition, we have reconstituted the activation of Cdc28p protein kinase in vitro. Addition of recombinant glutathione-S-transferase-CLN2 fusion protein to a CLN-deficient extract of G1-arrested *S. cerevisiae* cells resulted in up to a 200-fold activation of Cdc28p-associated histone H1 kinase activity. Activation was dependent upon time, temperature, ATP, and extract protein. These results provide direct evidence that G1 cyclins act by stimulating the protein kinase activity of Cdc28p, and open the door for further biochemical characterization of the activation and function of Cdc28p at the G1-S transition.

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2. Cross, F. (1988) *Mol. Cell Biol.* **8**, 4675-4684.
3. Hadwiger, J.A. et al. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6255-6259.

H 103 STUDIES OF THE MECHANISM AND TIME OF ACTION OF THE NOVEL AMINOCHROMONE ANTIPROLIFERATIVE U-67154, Laurence A. Erickson, Gregory J. Fici and Paul D. Bonin, Cardiovascular Diseases Research, Upjohn Laboratories, Kalamazoo, MI 49001

Intimal hyperplasia is the major feature of delayed restenosis after percutaneous transluminal coronary angioplasty. Polypeptide growth factors released at the site of balloon injury induce and maintain this response. Based on these findings, we initiated a program to discover novel compounds that would inhibit cell proliferation stimulated by a diverse array of mitogens. U-67154 was identified as the prototype of a series of aminochromones that reversibly inhibited DNA synthesis in and proliferation of BALB/c 3T3 fibroblasts and vascular smooth muscle cells stimulated by PDGF (EC_{50}, ~100 μM), FGF, EGF, and IGF-1, alone or in combinations. Many early signal transduction events associated with growth factor-induced cell proliferation were not affected by this compound. In contrast, ornithine decarboxylase (measured at five hours) and thymidine kinase (TK, measured at 18 hours), enzymes intimately associated with the induction of cellular proliferation, were inhibited by 50% and 87%, respectively. Moreover, TK activity was effectively inhibited when U-67154 was added to cells only during the three-hour interval between six and nine hours after serum stimulation. Neither of these inhibitory actions were attributable to direct neutralization of the enzymes or to generalized protein synthesis inhibition. Thus, the major point of action of U-67154 appears to occur in the G1 phase of the cell cycle. Current studies are focused on identifying the direct target(s) of U-67154 within this period. More potent analogues of U-67154 should prove useful in preventing intimal thickening in *in vivo* models of balloon angioplasty.

H 104 ISOLATION OF MAMMALIAN G1 REGULATORY GENES Jenö Gyuris, Erica Golemis, Helen Chertkov, and Roger Brent, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA, 02114 and Department of Genetics, Harvard Medical School

We are attempting to isolate mammalian genes that regulate the G1 phase of the cell cycle by using *S. cerevisiae*. We have developed a series of mammalian cDNA expression libraries, and a complementary series of yeast strains that allow us to employ three basic approaches to this problem. First, we are looking for mammalian genes that cause yeast to overcome the G1 specific arrest caused by α-factor. Second, we are searching for negative regulators of G1, using a selection strategy in which yeast are transiently arrested in G1 by pulsed expression of genes in a mammalian library and hence escape poisoning by a suicide substrate transiently provided in the medium. Third, we are using newly developed acid fusion cDNA libraries (Gyuris and Brent, unpublished) to probe for mammalian proteins that physically interact with human cdc2 and other cell cycle regulatory proteins (for example FUS3 and the G1 specific cyclins).

H 105 CYCLIN A ASSOCIATES WITH MULTIPLE PROTEIN KINASE SUBUNITS: CDK2 AND CDC2.

Frederick L. Hall[+], Stephen J. Elledge[¶], Ronald Richman[¶], Richard T. Williams[+], Naomi Lodgson[*], and J. Wade Harper[*], [+]Division of Orthopaedic Surgery, Childrens Hospital of Los Angeles, University of Southern California, School of Medicine, Los Angeles, California 90027, [*]Department of Biochemistry and [¶]Institute for Molecular Genetics, Baylor College of Medicine, Houston, Texas 77030.

Transitions in the eukaryotic cell cycle are controlled by the coordinate actions of the $p34^{cdc2}$ protein kinase and its regulatory subunits, cyclins. While G1-S and G2-M cyclins interact with a single p34 protein to regulate these transitions in yeast, the situation is more complex in higher eukaryotes. Recently we identified a new human p34 homolog, *CDK2* (cell division kinase) by complementation of a *cdc28*-4 mutation in *S. cerevisiae* using a YES human cDNA expression library. *CDK2* is 66% identical to *CDC2Hs* and 89% identical to the *Xenopus* Eg1 gene, forming a distinct sub-family of *CDC2* related protein kinases. In activated peripheral blood lymphocytes, increases in *CDK2* and cyclin A message levels are observed as early as 16h post-stimulation, prior to S-phase, while increases in *CDC2Hs* message are detected much later (24h). In Hela cells, cyclin A is found associated with *CDK2* but not with *CDC2*. Cyclin A associates with a less abundant form of *CDK2* which migrates slightly faster (~33K) than the major form (~34K) on SDS gels. In contrast, both cyclin A/*CDK2* and cyclin A/*CDC2* complexes are present in mouse FM3A cells and in Wilms tumor tissue, suggesting that cells in different developmental or transformed states may have distinct cell cycle regulation.

H 106 STRUCTURE AND CHARACTERIZATION OF THE HUMAN p58 PROTEIN KINASE GENE AND ITS PRODUCT, Vincent J. Kidd[1,2], Peter Eipers[1,3], Jialing Xiang[1,2], José Grenet[1], and Jill M. Lahti[1], [1]Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN 38101 and the Departments of [2]Cell Biology and [3]Medical Genetics, University of Alabama at Birmingham, Birmingham, AL 35294

The structure, chromosome localization, and cellular activity of a novel human cell division control-related protein kinase, p58, have been determined. The gene is encoded by 13 exons that span approximately 11 kb on human chromosome 1p36. Deletion of this region of human chromosome 1 has been implicated in a number of tumors, including malignant melanoma and neuroblastoma. Gene structure also demonstrates that unique amino- and carboxy-terminal domains of the protein are encoded by exons that are distinct from the cell division control-related protein kinase domain, supporting the hypothesis that this gene arose by gene fusion. Analysis of p58 protein kinase activity in human cells has shown that, although steady-state protein levels are invariant, p58 mRNA levels, kinase activity and phosphorylation state fluctuate dramatically during the cell cycle. Steady-state p58 mRNA levels parallel $p34^{cdc2}$ mRNA expression in both aphidicolin/thymidine and nocodazole/thymidine blocked and released cells. In contrast, p58 protein kinase activity is maximal at both the G_1/S boundary and during G_2/M-phase of the cell cycle in similarly blocked and released cells, while $p34^{cdc2}$ protein kinase activity peaks only during mitosis. These peaks in protein kinase activity are coincident with changes in p58 phosphorylation. When p58 protein kinase activity is high, the protein is minimally phosphorylated on serine residues, whereas low p58 protein kinase activity coincides with increased phosphorylation of serine residues. These studies, coupled with previous studies demonstrating that elevated p58 expression leads to negative regulation of cell growth, suggest that this protein kinase may play an important role in normal cellular growth.

H 107 FEEDBACK OF REPLICATION ON MITOSIS: IN VITRO CELL CYCLE ARREST USING ARTIFICIAL DNA TEMPLATES, Sally Kornbluth, Carl Smythe, and John Newport, Department of Biology, University of California, San Diego, La Jolla CA 92093.

In cell-free extracts of Xenopus eggs which oscillate between S and M phases of the cell cycle, the onset of mitosis is blocked by the presence of incompletely replicated DNA. We show here that several artificial DNA templates (M13 single stranded DNA, double stranded plasmid DNA) can trigger this feedback of replication on mitosis.

Single stranded DNA, which replicates much more efficiently than double stranded plasmid in the Xenopus extracts is also more effective at inhibiting the onset of mitosis, implicating ongoing replication, rather than the presence of unreplicated DNA per se in the generation of the feedback signal. Furthermore, we have shown that low levels of M13 single stranded DNA and high levels of double stranded plasmid DNA can elevate the tyrosine kinase activity responsible for phosphorylating $p34^{cdc2}$, thereby inactivating maturation promoting factor (MPF) and inhibiting entry into mitosis. This constitutes a simplified system with which to study the signal transduction pathway from the DNA template to the tyrosine kinase responsible for inhibiting $p34^{cdc2}$ activity.

H 108 MAPPING THE FUNCTIONAL DOMAINS OF A NOVEL CELL DIVISION CONTROL-RELATED PROTEIN KINASE, Jill M. Lahti[1], Lucie S. Heath[2], and Vincent J. Kidd[1,2], [1]Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN 38101 and [2]Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35294

A novel cell division control-related protein kinase, p58, has previously been isolated and characterized from both human and mouse. Minimal overexpression of this protein kinase in eukaryotic cells results in a cell cycle delay at late telophase and an increased frequency of abnormal cytokinesis. The p58 protein kinase contains two structural domains not found in either $p34^{cdc2}$ or $p33^{cdk2}$. One of these domains, an amino-terminal region of 76 amino acids, contains sequences that may specify calmodulin binding and nuclear localization. We have confirmed that calmodulin binds to this amino-terminal domain of the protein kinase. Site-specific and deletion mutants of the p58 protein kinase were created in an attempt to understand p58 function and regulation. Deletion of the amino-terminal domain results in a protein kinase that produces a unique cellular phenotype when expressed in eukaryotic cells. More specifically, the cells have a rounded-up morphology and are small in size, but they are no longer delayed at late telophase and cytokinesis is normal. In contrast, expression of p58 kinase negative mutants in these same cells does not alter cellular morphology or growth. These results suggest that the amino-terminal domain of the p58 protein kinase is essential for producing the changes in cell cycle progression and cytokinesis previously observed. These studies also suggest that additional regions of the p58 protein kinase, most likely the carboxy-terminal domain, are important for the observed morphological changes in cell shape and size.

H 109 DIFFERENT G1 CYCLINS CONTROL CELL CYCLE COMMITMENT IN MOTHER AND DAUGHTER CELLS OF THE BUDDING YEAST *Saccharomyces cerevisiae*, Daniel J. Lew, Nicholas J. Marini, and Steven I. Reed, Scripps Research Institute, Department of Molecular Biology, MB7, 10666 N. Torrey Pines Road, La Jolla, CA 92037

Growth of S. cerevisiae cells by budding gives rise to asymmetric progeny cells: a larger "mother" cell and a smaller "daughter" cell. The mother cell transits a brief G1 phase before forming a new bud and beginning DNA replication. The daughter cell stays in G1 for a longer period, growing in size before initiating a new cell cycle. We show that the timing of cell cycle initiation in mother and daughter cells is governed by distinct G1 cyclins. In daughter cells, transcription of CLN1 and CLN2 is induced in a size dependent manner, and these cyclins are necessary for the normal timing of cell cycle initiation. CLN3 is not required in daughter cells, but is crucial for mother cells, in which the G1 phase is much longer in the absence of this cyclin.

H 110 INSERTIONAL MUTAGENESIS OF A MAMMALIAN GENE INDUCED BY CELL GROWTH ARREST PROLONGS THE CELL CYCLE BY DELAYING THE G1 TO S TRANSITION, S. Lin-Chao[1], A.C.Y. Chang[2], A. Yarden[2], and S.N. Cohen[2]. Institute of Molecular Biology[1], Academia Sinica, Taipei and Department of Genetics[2], Stanford University, Stanford CA 94305.

The use of retrovirus-derived *lac Z*-based reporter gene ("promoter trap") vectors for the detection of mammalian genes induced by cell growth arrest in the Go state has been decribed previously (D.G. Brenner et al, PNAS 86:5517, 1989). Here we report that a retroviral insertion into one chromosomal allele of each of two growth-arrest-specific *(gas)* genes in cultured NIH 3T3 cells leads to prolongation of the cell cycle from 15 to >20 hours during normal growth. The observed prolongation was associated with an increase from 46% to 55-60% in the fraction of cells in G1, suggesting that entry of cells into S phase is delayed by the mono-allelic insertional inactivation of these *gas* genes, and implying that the cellular concentration of the gene products may affect the transition of cells from G1 to S. Northern blotting confirmed that the *lacZ* reporter gene is located within a regulated transcript in cells containing one of the inserts. Analysis of cloned genomic DNA containing inactivated *gas* genes and of corresponding cDNA shows that *lacZ* is spliced to 5' exonic segments whose sequence shows no similarity to genes previously identified as having a role in regulation of the cell cycle or cell growth. The mechanism by which the insertional mutations may affect the G1/S transition is under investigation.

H 111 GENOMIC ORGANIZATION, CHROMOSOMAL LOCALIZATION, AND INDEPENDENT EXPRESSION OF HUMAN CYL (CYCLIN D) GENES, A. Thomas Look, Toshiya Inaba, Hitoshi Matsushime, Marcus Valentine, Martine F. Roussel and Charles J. Sherr, Departments of Hematology-Oncology, Tumor Cell Biology, and Howard Hughes Medical Institute, St. Jude Children's Research Hospital, Memphis, Tennessee 38105 and Departments of Pediatrics and Biochemistry, University of Tennessee College of Medicine, Memphis, Tennessee 38163

Murine cDNA clones for three cyclin-like (CYL) genes that are normally expressed during the G_1 phase of the cell cycle were used to clone the cognate human genes. Bacteriophage and cosmid clones encompassing five independent genomic loci were partially sequenced and chromosomally assigned by an analysis of somatic cell hybrids containing different human chromosomes and by fluorescence in situ hybridization to metaphase spreads from normal peripheral blood lymphocytes. The human CYL1 gene was assigned to chromosome 11q13, the CYL2 gene to chromosome 12p13, and the CYL3 gene to chromosome 6p21. Pseudogenes containing sequences related to CYL2 and CYL3 mapped to chromosomes 11q13 and 6p21, respectively. Partial nucleotide sequence analysis of exons within each gene revealed that the authentic human CYL genes are more related to their mouse counterparts than to each other. These genes are ubiquitously transcribed in human tumor cell lines derived from different cell lineages, but are independently, and in many cases, redundantly expressed. The complex patterns of expression of individual CYL genes and their evolutionary conservation across species suggest that each family member may play a distinct role in regulating cell cycle progression.

H 112 IDENTIFICATION OF CELL CYCLE REGULATORY KINASES (cdc2 AND cdk2) AND ASSOCIATED PROTEINS IN NORMAL HUMAN T LYMPHOCYTES, Joseph J. Lucas, Naohiro Terada, Agota Szepesi, Colm Kelleher, Joanne Domenico and Erwin W. Gelfand, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206

Human T cells isolated from peripheral blood provide a population of normal cells in the G_0 phase of the cell cycle. Upon stimulation with various agents (such as phorbol ester and ionomycin) the cells will synchronously enter the cell cycle and proliferate. The system is proving of particular value for studying events in the earliest phases of cell cycle passage, such as the G_0/G_1 transition and passage through the restriction point (R), since the cells initially lack molecules required for late G_1 and later phases, such as the $p34^{cdc2}$ kinase and cyclin A. These latter molecules were first detected in activated T cells at about 30 hr after stimulation, shortly before S-phase entry. It was noteworthy that significant synthesis of these molecules began after substantial phosphorylation of the $p110^{Rb}$ protein had occurred, an observation suggesting that $p34^{cdc2}$ may not be the main kinase for $p110^{Rb}$. In these experiments, $p34^{cdc2}$ was detected by immunoblotting using a specific antibody directed against the C-terminus of the protein. Using an antibody directed against the common "PSTAIRE" region shared by all cdc2-homologues, a protein with a mobility slightly lower than hypophosphorylated $p34^{cdc2}$ was detected, even in resting cells. At later times in G_1 phase, a higher mobility species with an apparent molecular weight of 33 kd could also be seen using this antibody as probe. The interrelationships of these proteins are now being dissected using techniques of two dimensional gel electrophoresis and peptide mapping. Also, using an oligonucleotide probe containing sequences shared by the human cyclin D and rodent CYL family of genes, transcripts were detected at this early stage of T-cell activation, well before the synthesis of $p34^{cdc2}$ and cyclin A. Cloned sequences are being isolated from a cDNA library prepared from T cells at 8 hr after activation.

H 113 A MAMMALIAN PROTEIN-SERINE/TYROSINE KINASE RELATED TO THE nimA CELL CYCLE REGULATOR. Lee Mizzen, Ken Letwin, Yaacov Ben-David, Benny Motro, Alan Bernstein and Tony Pawson. Division of Molecular and Developmental Biology, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave., Toronto, Ontario, Canada. M5G 1X5.

Using a functional screen to identify new tyrosine kinsases, a mouse erythroleukemia cell cDNA expression library was probed with anti-phosphotyrosine antisera. This approach allowed the isolation of several cDNAs encoding proteins identical or very similar to known protein-tyrosine kinases. However, two isolated cDNAs, designated clk and nek, encode proteins most closely related to kinases involved in regulating progression through the cell cycle and contain motifs generally considered diagnostic of protein-serine/threonine kinases. The characterization of clk has recently been published (Ben-David et.al.,(1991) EMBO J.10. 317-325) and the present abstract focuses on the characterization of nek. The nek gene is 2.3 kb in length and encodes a protein of 774 amino acids (88.4 kDa) with a pI of 10.22. The nek protein is comprised of an N-terminal catalytic domain and a large "tail" domain at the C-terminus. A search of the GENEMBL database revealed nek was most similar to the nimA protein, a serine/threonine kinase involved in regulating the G2-M transition in *Aspergillus nidulans*.

When expressed in bacteria, the nek protein exhibits serine/threonine and tyrosine kinase activity, as judged by both anti-PTyr immunoblotting and hydroxyamino acid analysis of bacterial lysates. In mouse tissues, northern analysis revealed that a 6 kb transcript was expressed at low levels in most tissues examined. Nek expression was highest in testes where an additional 4 kb transcript was present. In vivo, RNA in situ analysis indicated that nek expression was localized to the dorsal root ganglia of the developing mouse embryo (13.5 day) and to the testes, spleen and ovary of adult mice.

H 114 THE ROLE OF CYCLIN-DEPENDENT KINASE-2 (CDK2) IN MAMMALIAN CELL CYCLE CONTROL. David O. Morgan, Jody Rosenblatt, Yong Gu, and Dipty Desai, Department of Physiology, University of California, San Francisco CA 94143-0444.

We have identified and characterized human CDK2, a closely related homologue of the cell cycle regulatory protein kinase CDC2. Using two experimental systems, we find that the Histone H1 kinase activity of CDK2, like that of CDC2, is dependent on cyclins: (i) CDK2 is activated by transient co-expression with human cyclins A or B1 in COS-7 cells, and (ii) baculovirus-derived CDK2 is inactive as a monomer but is activated by mixing with human cyclins A or B1 in vitro. To explore the regulation of CDK2 in the cell cycle, we have developed specific anti-peptide antibodies against the C-terminal sequences of human CDK2 and CDC2. Analysis of kinase activity in immunoprecipitates of HeLa cell lysates demonstrates that CDK2, like CDC2, undergoes periodic activation. However, the patterns of CDK2 and CDC2 activation are different. CDC2 activity peaks during mitosis (in parallel with Cyclin B levels), while CDK2 activity peaks during the S and G2 phases and declines during mitosis (in parallel with Cyclin A levels). Similar patterns of CDK2 activation are seen in mouse 3T3 cells stably expressing epitope-tagged CDK2. Activated CDK2 in HeLa and 3T3 cells is found in multiple high molecular weight complexes, and CDK2 in one of these complexes is associated with Cyclin A. In addition, CDK2 activation is accompanied by phosphorylation of the CDK2 subunit in these complexes. These observations suggest that CDK2 and CDC2, in association with different cyclins, are involved in the control of distinct cell cycle events.

H 115 EXPRESSION AND DISTRIBUTION OF THE JUN PROTEINS DURING THE CELL CYCLE, Curt M. Pfarr, Fatima Mechta, Giannis Spyrou, Vassilis Doucas, and Moshe Yaniv, Oncogenic Viruses Unit, Department of Biotechnology, UA 1149 CNRS, Institute Pasteur, Paris.

The Jun family of oncoproteins (c-Jun, JunB, and JunD) function as transcriptional activators by interacting with other nuclear factors and directly binding to DNA. While sharing considerable structural homology the three Jun proteins are differentially regulated during the cell cycle and have distinct modes of DNA binding and transcriptional activation. When quiescent NIH-3T3 cells are stimulated to grow, mRNA levels for both c-Jun and JunB rise sharply then fall to undetectable levels by S-phase and remain very low. In contrast, JunD mRNA levels undergo only a small increase during Go-G1, fall to a minimum by S-phase then rise slowly as the cells grow confluent. Protein levels for c-Jun and JunB rise to high levels by 2 hours after stimulation and remain elevated throughout the first cell cycle, decreasing afterwards. JunD protein levels, however, remain relatively constant. These induction kinetics are likely to reflect different functions for each of the Jun proteins during the cell cycle. To further explore these differences we are investigating the cellular distribution of each Jun protein by immunolocalization and cell fractionation analyses.

H 116 PHOSPHORYLATION OF MAP KINASE DURING MEIOSIS IN XENOPUS OOCYTES, James Posada, Jonathan A. Cooper, Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104.

We have cloned Xp42, a mitogen-activated protein (MAP) kinase from Xenopus oocytes. MAP kinase is activated by phosphorylation at the time of germinal vesicle breakdown (GVBD) during progesterone-induced meiotic maturation. The sites of phosphorylation of Xp42 have been mapped to T188 and Y190, and enzymatic activity requires both T188 and Y190 to be phosphorylated. Expression of a kinase-inactive mutant (K57R) in Xenopus oocytes has shown that both T188 and Y190 are phosphorylated in trans by kinases present in Xenopus oocytes since the K57R mutant is phosphorylated at meiosis in progesterone-treated oocytes. In addition, T188V and Y190F mutants are both phosphorylated during meiosis in Xenopus oocytes suggesting that the threonine and tyrosine phosphorylations are independent events. Replacing T188 with a charged residue, aspartic acid, does not effect tyrosine phosphorylation. A carboxy-terminal truncation mutant which is lacking the C-terminal 34 amino acids is tyrosine phosphorylated at approximately 10% of WT suggesting that the C-terminus stabilizes a conformation which renders Xp42 a substrate for a tyrosine kinase. Our data are consistent with the notion of MAP kinase responding to signals being transmitted separately by tyrosine and threonine kinases.

H 117 CELLULAR GROWTH POTENTIAL IS DIRECTLY RELATED TO AP-1 ACTIVITY IN NORMAL, SENESCENT AND TRANSFORMED HUMAN FIBROBLASTS. Karl T. Riabowol Department of Medical Biochemistry, University of Calgary, Health Sciences Centre, Calgary, Alberta, T2N 4N1

Transformed human fibroblasts grow rapidly in culture and unlike normal human diploid fibroblasts (HDFs), are unable to enter a quiescent state when deprived of growth factors or upon reaching confluence. Normal HDFs also display a limited replicative capacity which is characterized by a gradual decrease, over many population doublings, in their ability to respond fully to extracellular stimuli. This decrease culminates in old HDFs entering a state termed cellular senescence in which growth is no longer elicited by factors which are normally mitogenic. We have shown recently that AP-1 binding factors are required for progression through the G_1 portion of the cell cycle by selectively inactivating these binding factors in living cells by needle microinjection. Moreover, as normal HDFs begin to age they show a striking decline in total AP-1 activity as measured *in vivo* by reporter constructs and *in vitro* by mobility shift assays and DNA precipitation assays. We now extend this work to show that rapidly growing transformed human fibroblasts contain considerably more AP-1 activity than normal HDFs, despite the observation that synthesis of Fos and Jun in response to extracellular stimuli is comparable in normal and transformed cells. In addition, DNA precipitation assays indicate that modification of the Fos protein is markedly different in transformed, normal and senescent human fibroblasts. Thus, modification of the Fos protein and total AP-1 binding activity are directly correlated to growth potential, with the most rapidly proliferating cell types (transformed human fibroblasts) having large amounts of AP-1 activity, and Fos protein which is less heavily modified than that isolated from normal HDFs.

H 118 INSULIN AND PROGESTERONE INCREASE ^{32}P-LABELING OF PHOSPHOLIPIDS, AND DAG AND IP3 MASS DURING INDUCTION OF MEIOSIS IN XENOPUS OOCYTES. Bradley J. Stith, Marc Goalstone, Chris Jaynes, Sally Silva, Biology Department, University of Colorado at Denver, Denver, CO 80217-3364.

Insulin and progesterone induce Xenopus oocytes to enter prophase of meiosis after a 3-6 hr induction period. Progesterone induces an increase in ^{32}P-labeling of phosphatidylcholine and phosphatidylinositol at 15 min after hormone addition and again just before cells enter prophase. Labeling of phosphatidylserine and phosphatidylethanolamine is not altered. Insulin changes labeling of all of the phospholipids measured. Progesterone and insulin both increase DAG levels (25-60%) with two peaks occurring at 15 min and just before entry into meiosis. As PIP2 breakdown produces inositol 1,4,5-trisphosphate (IP3), we measured IP3 by receptor assay. Both insulin and progesterone induce two peaks in IP3 mass; the first is at about 15 min and a second slow increase begins at about 0.5 GVBD50 (i.e., 2 hrs after progesterone and 3 hrs after insulin). The peaks represent an increase from about 64 to 100 nM IP3. Insulin is probably acting through the IGF-1 receptor as insulin concentrations greater than 50 nM are required to induce meiosis or increase IP3 or DAG. We conclude that only 0.5 % of the DAG produced (about 20 pmol) is from PIP2 breakdown since the IP3 increase during these periods is only 0.1 pmol.

H 119 EFFECTS OF RAPAMYCIN ON CELL CYCLE PROGRESSION, Naohiro Terada, Joseph J. Lucas, Richard A. Franklin, Agota Szepesi, Joanne Domenico and Erwin W. Gelfand, Division of Basic Sciences, Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver CO 80206

Rapamycin (RAP) is a novel macrolide with potent immunosuppressive activity. In contrast to cyclosporin A and FK506, RAP appears to disrupt a transduction pathway after cytokine-cytokine receptor interaction. We have attempted to further localize the point in the cell cycle where RAP acts to inhibit T-cell proliferation. First, PHA-stimulated human T cells were analyzed. RAP blocked cell cycle progression at the early to mid G1 phase in terms of cellular RNA content. This blockade was after IL2-IL2 receptor interaction, but before $p110^{Rb}$ phosphorylation, and $p34^{cdc2}$ and cyclin A synthesis, all of which occurred at mid to late G1 phase in PHA-stimulated T cells. Second, cell cycle progression after IL2-IL2 receptor interaction was analyzed using the IL2 dependent T-cell line Kit225, or competent human T cells prepared by brief exposure to phorbol ester and calcium ionophore. In both conditions, the cell cycle was apparently arrested by RAP shortly after IL2-IL2 receptor or IL4-IL4 receptor interactions in terms of cellular RNA content. In IL-2 stimulated Kit225 cells, RAP did not affect MAP2 kinase activity or expression of c-fos and c-myc mRNAs, but did inhibit $p110^{Rb}$ phosphorylation and $p34^{cdc2}$ phosphorylation. Recent studies suggest that a homologue of $p34^{cdc2}$, p33, may play a role in earlier phases of cell cycle progression. The possible effect of RAP on p33 and its dephosphorylation are now being investigated. These findings further define the point of action of RAP and suggest a set of molecular events on which to focus further studies.

H 120 LINKING GROWTH FACTORS TO CELL CYCLE CONTROL: G1 CYCLINS, Kwang-Ai Won and Michael Z. Gilman, Cold Spring Harbor Laboratory, P.O.Box 100, Cold Spring Harbor, N.Y. 11724

It is not well understood how the initial intracellular signalling events triggered by growth factors are linked to the regulatory machinery that controls cell cycle progression in mammalian cells. To test the hypothesis that transcriptional regulation of G1 cyclins is one of mechanisms involved in this connection of growth factor signals to cell cycle control, we determined if transcriptional activity of human D-type cyclins changes in response to serum in primary human fibroblasts. The levels of cyclin D1 and D3 RNAs increase gradually and peak at about 12 hour after serum stimulation. To identify specific growth factor(s) responsible for the induction of cyclin genes, cells were treated with various agents following serum starvation. We found that both PDGF and phorbol ester strongly induced D1 and D3 mRNAs. Interestingly, when [^{3}H]thymidine incorporation was measured to confirm the G1 to S transition, PDGF but not phorbol ester led to the induction of DNA synthesis. Therefore, transcriptional activation of these cyclin genes is not sufficient to drive cells from quiescence through G1 into S phase. The finding that the two cyclin genes respond to the same growth factor raises questions concerning common regulatory elements for coordinate gene expression. Localization of regulatory elements responsive to growth factor signals are underway and will be presented.

H 121 THE mos PROTOONCOGENE PRODUCT AND THE REQUIREMENT FOR PROTEIN SYNTHESIS DURING XENOPUS OOCYTE MATURATION, Nelson Yew[1], Michael L. Mellini[2], and George F. Vande Woude[1], [1]ABL-Basic Research Program, [2]Program Resources, Inc., NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702-1201

Translation of the maternal mos protooncogene mRNA is specifically induced by the natural hormone, progesterone, during *Xenopus* oocyte maturation. We and others have shown that translation of the endogenous mos mRNA is essential for both the first and second meiotic divisions in *Xenopus* oocytes [Daar et al. (1991) J. Cell Biol., 114:329-335; Kanki and Donoghue (1991) PNAS 88:5794-5798]. The mos product is also required in unfertilized eggs since it is an active component of cytostatic factor [Sagata et al. (1989) Nature 342:512-518] an activity responsible for the arrest of oocyte maturation at metaphase II of meiosis. Moreover, injection of translatable mos RNA into oocytes induces their maturation in the absence of the normal hormonal stimulus [Sagata et al. (1989) Science 245:643-646]. This suggests that the mos protein can serve as an "initiator" of oocyte maturation, originally conceived as a protein whose synthesis is necessary to trigger the conversion of latent, inactive M-phase promoting factor (MPF) into its active form. It is possible that other proteins in addition to mos are synthesized de novo in response to progesterone and are required for meiosis. We are currently investigating whether the mos protein can induce germinal vesicle breakdown and the activation of MPF in the absence of any protein synthesis during oocyte maturation.

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H 122 DIFFERENTIAL TYROSINE PHOSPHORYLATION OF NOVEL SUBSTRATES INDUCED BY FGF-1 IN EARLY AND LATE G_1 PHASE, Xi Zhan, Xiaoguo Hu, Stanley Friedman and Thomas Maciag, Laboratory of Molecular Biology, American Red Cross, Rockville, MD 20855

The stimulation of DNA synthesis in Balb/c 3T3 cells by fibroblast growth factor (FGF)-1 requires at least 12 hr expqsure of the cells to the mitogen as demonstrated by FGF-1 depletion studies and by disruption of the interaction of the ligand and its receptor. In addition, we studied the induction of FGF-1-induced phosphotyrosyl proteins in whole cell lysate prepared from cells at different stages of the cell cycle. At least three new phosphorylated proteins were detected. Interestingly, a p60 appeared at 6 hr after stimulation, and a protein with an apparent molecular weight (M_r) of 82 Kd was detected at 10 hr after the addition of FGF-1. Another phosphorylated polypeptide was also observed with a M_r of 150 Kd at early time points and was present at a reduced level at later time points. During the later period, another polypeptide with a M_r of 170 Kd was also observed. Interestingly, a protein with a M_r of 130 Kd also appeared to be phosphorylated at the later time points. In addition, p90, which has previously been shown to be a major substrate for the FGF receptor tyrosine kinase, was maximally stimulated at 30 min. Two hr after the addition of FGF-1, the level of p90 phosphorylation was reduced approximately 20%, and this level of p90 phosphorylation was maintained during the rest of the cell cycle. Cycloheximide studies demonstrated that the stimulation of phosphorylation of p82, p170, and p60, but not p90, requires new protein synthesis. Since it has been possible to co-precipitate p82 and p60 with an FGF receptor (*flg*) antiserum, these data argue that the FGF receptor may interact with different and unique substrates both early and late in the G_1 phase of the cell cycle.

Growth Factors

H 123 Cytokines regulating the proliferation of culture-derived macrophages in cytokine-free, serum-free medium. Bennett S, Por S.B, Stanley E.R and Breit S.N. Centre for Immunology, St.Vincent's Hospital. Sydney Australia. 2010.

Until recently, macrophages were believed to be nonreplicating end cells. However it is now known that local replication does occur and is clearly important in regulating tissue macrophage numbers. The factors which are mainly responsible for replication of monocyte precursors are thought to include M-CSF, GM-CSF, IL-3, and perhaps IL-6 and TNF-a. The factors which are responsible for macrophage replication in tissues are largely unknown.

A cytokine-free, serum-free system which supports the long term culture and proliferation of human peripheral blood monocytes has been established. The cells maintained in this system have similar properties to tissues macrophages. Cell proliferation was found to occur during the first two weeks of culture with maximal tritiated thymidine incorporation occurring at days 3 and 4. Cells maintained for 5 weeks in serum-free medium (SFM) secreted high levels of TNF-a and IL-6 during week one and GM-CSF during the third week. Additionally, these cells constitutively produced large amounts of M-CSF in the absence of any exogenous stimuli or LPS contamination. Maximal production of M-CSF (1809 pg/mL) occurred during the first week. To ascertain if this secreted M-CSF was responsible for proliferation, a high affinity antibody directed against M-CSF was added to the cultures at various time points. This antibody did not inhibit replication suggesting that proliferation does not depend entirely on secreted M-CSF. The addition of an antibody directed against IL-3 was found however to partially inhibit proliferation over the first 4 days. Further studies are currently being undertaken to elucidate the role of these cytokines in macrophage proliferation.

H 124 PROLIFERATIVE RESPONSE OF PRIMARY MOUSE COLONIC EPITHELIAL CELLS TO GROWTH FACTORS AND BILE ACIDS.
Christina Branting and Joseph J. Rafter, Department of Medical Nutrition, Karolinska Institutet, Huddinge Univ. Hospital F60, NOVUM, S-141 86 Huddinge, Sweden.
Because diet has been implicated in the causation of colon cancer and dietary factors may exhibit tumor promoting activity and affect growth of the colonic epithelium, primary cultures of mouse colonic epithelial cells have now been established and the effect of growth factors, a model tumor promoting agent, i.e. 12-O-tetradecanoylphorbol 13-acetate (TPA) and the secondary bile acid deoxycholic acid studied. Primary cultures, derived in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (FBS), 0.5µg/ml hydrocortisone, 5µg/ml insulin, 5µg/ml transferrin and 5ng/ml selenium, exhibited a typical epithelial morphology and uniformely expressed keratins and moreover, a major fraction of the cells stained for ß-galactosidase. The cultures could be subcultivated as islets of cells using dispase while maintaining their epithelial morphology and the above functions for at least three passages. Using cells kept in a minimal maintenance medium, i.e. growth medium containing only 0.5% FBS, the proliferative effect of the above factors was measured by autoradiographic analysis of ^{3}H-thymidine incorporation. Epidermal growth factor (10 and 30ng/ml) and insulin (10 and 100µg/ml) each markedly increased the proportion of labeled cells above the background level of <1 percent; together these factors acted in an additive manner and constituted 70 to 80% of the maximal growth stimulation (28% labeled cells) obtained in the presence of complete growth medium. Deoxycholic acid, 25 and 100µM, i.e. at normal human fecal water concentrations, also stimulated proliferation and increased the fraction of labeled cells to 4 and 7 percent, respectively. The proliferative effect of each of these factors was confirmed by counting the number of mitotic figures in cultures growth-arrested by colchicine treatment. Finally, TPA (between 1 to 100nM) increased the fraction of labeled cells to maximally 10 percent, an effect that was blocked by an inhibitor of protein kinase C, i.e. staurosporine. Immunocytochemical analysis showed production of the c-fos protein already 1h following exposure to TPA. However, neither the effect of staurosporine on cell growth nor production of the c-fos protein was demonstrated for the potential tumor promoting agent deoxycholic acid. Taken together, the present study demonstrates that mouse colonic epithelial cells can be established in both primary and transfer cultures, and moreover, respond to various growth factors and potential tumor promoting agents.

H 125 **MECHANISM OF CROSS STIMULATION BETWEEN HORMONE-DEPENDENT AND INDEPENDENT BREAST CANCER CELL LINES.**
V. Cappelletti, P.Miodini, C.Coradini, G. Di Fronzo. Istituto Nazionale Tumori, Milano, Italy.
The hormone-dependent breast cancer cell line MCF-7 and the hormone-independent MDAMB-231 were co-cultured using the transwell system in a serum- free medium. When the MCF-7 cells were grown in the presence of transwells containing MDAMB-231 cells, a stimulation of growth ranging from 70 to 150%, as a function of culture conditions, was observed in the presence and in the absence of estradiol. Addition of EGF (10 ng/ml) and bFGF (1 ng/ml) stimulated the growth of single and co-cultures, but the growth ratio of the two systems was maintained. Conversely, following treatment with IGF-1 and TGF alpha, a stimulation of growth of single- and co-cultures was still observed, but the proliferative advantage in co-cultures compared to single cultures disappeared. These findings suggest that these growth factors are involved in the paracrine stimulation observed in our experiments. This hypothesis is supported by evidence that addition of a monoclonal antibody against TGF-alpha, which was previously demonstrated to block TGF-alpha-induced growth stimulation, completly abolished the differential growth of single- and co-cultures. Similarly, the antibody was able to completely inhibit cell growth stimulation exerted by estradiol in MCF-7 cells.

H 126 A CELL SURFACE TRANSMEMBRANE GLYCOPROTEIN WITH GROWTH FACTOR-LIKE ACTIVITY,
Kermit L. Carraway, III, Pamela M. Guy, Richard A. Cerione, Coralie A. C. Carraway and Kermit L. Carraway, Cornell University, Ithaca, NY 14853, and Univ. of Miami School of Medicine, Miami, FL 33101.
Molecular cloning and sequencing of ASGP-2, a cell surface glycoprotein overexpressed in rat 13762 ascites adenocarcinoma cells, has shown that this protein contains two EGF-like repeats anchored to the membrane via a transmembrane helix. One of the EGF-like repeats has significant homology to known activators of the cell surface receptor for epidermal growth factor. We have tested *in vitro* the possibility that ASGP-2 could act as a growth factor by examining its interaction with the EGF receptor in isolated plasma membranes from A431 human epidermoid carcinoma cells. We found that ASGP-2 was a potent inhibitor of the binding of [^{125}I]EGF to membrane receptors. Moreover, ASGP-2 was capable of activating the protein tyrosine kinase activity of the EGF receptor. These results suggest the possibility that such cell surface proteins could act to regulate cell growth via autocrine or juxtacrine mechanisms.

H 127 DIFFERENTIAL EFFECTS OF GROWTH FACTORS ON MITOGENESIS AND TGFα AUTOCRINE REGULATION IN HUMAN ESOPHAGEAL CARCINOMA CELLS,
Shan-Chun Chen[1,3], Chen-Kung Chou[1,2], Chungming Chang[1,2] and Cheng-po Hu[1,2], [1]Graduate Institute of Microbiology and Immunology, National Yang-Ming Medical College, [2]Department of Medical Research, Veterans General Hospital, [3]Institute of Biomedical Sciences, Academia Sinica, Taipei, R.O.C.
The growth regulatory effects of EGF, TGFα, IGF-I, insulin, and PDGF on a human esophageal carcinoma cell line CE48T/VGH were evaluated. Under serum-free conditions, these mitogens promoted 2.8- to 3.9- fold increase of cell proliferation. Dose response studies and ^{125}I-ligand binding competition assays revealed that EGF and TGFα equipotently stimulated cell growth through interactions with surface EGF/TGFα receptors, while the proliferative signal of insulin is likely mediated through surface IGF-I receptors. PDGF-BB also promoted the growth of CE48T/VGH. Dose response studies using different PDGF isoforms suggested the existence of type β-like PDGF receptors in this epithelial carcinoma cell. Stimulation responses in TPA-pretreated cells suggested that the proliferative signals of PDGF, EGF, TGFα and IGF-I were transduced through protein kinase C (PKC) — dependent and — independent pathways. The cell cycle-related "competent genes", c-*fos*, and c-*jun*, were strongly induced by EGF and TGFα, but much weakly and transiently induced by IGF-I and PDGF. Furthermore, TGFα rapidly autoinduced TGFα and EGF/TGFα receptor gene expression with kinetics similar to that induced by TPA. IGF-I and PDGF also transmodulated TGFα autocrine loop, although with delayed kinetics. Our studies demonstrated the involvement of multiple growth factors in the control of proliferation for CE48T/VGH cells, whereas the individual growth factor may regulate different intracellular events through various post-receptor signaling pathways.

H 128 THE CENTRAL PORTION OF GASTRIN RELEASING PEPTIDE AND SOMATOSTATIN MEDIATE SPECIFIC INTERACTIONS WITH NON-RECEPTOR PROTEINS FROM CELLS AND SERUM. **Bradford O. Fanger, Elizabeth A. Cashman, Arlene C. Wade and Alan D. Cardin. Marion Merrell Dow Research Institute, Cincinnati, OH 45215**

The carboxyl-terminal domain (^{20}His-Trp-Ala-Val-Gly-His-Leu-Met-NH_2) of gastrin releasing peptide (GRP) is conserved and mediates GRP binding to its cellular receptor. The central portion (^{15}Tyr-Pro-Arg-Gly) of GRP is also conserved, suggesting that it may have a physiological function. Proteins are described which bind the central portion of GRP and to a similar region (^{7}Phe-Trp-Lys-Thr) in somatostatin (SS). These tetrapeptide sequences consist of aryl, nonpolar, positively charged, and uncharged amino acids. ^{125}I-GRP and ^{125}I-SS were chemically cross-linked to a 120 kDa protein in Triton extracts of Swiss 3T3 cells with bis(sulfosuccinimidyl) suberate. The labeling was blocked by unlabeled GRP, GRP(14-27) and SS, while peptides lacking these sequences, bombesin, [Tyr^{4}]bombesin, AcGRP(20-27), and the SS analogs cyclo-SS and RC-160, did not block labeling at 100 nM concentrations. Cross-linking studies also identified a 63 kDa protein in fetal calf serum and in a bovine serum albumin (BSA) preparation. This protein had a specificity for these tetrapeptide regions that was similar to but not identical with that of the 120 kDa protein. Binding of ^{125}I-GRP and ^{125}I-SS to the 63 kDa protein in BSA was inhibited by GRP, GRP(14-27), and SS, but not by AcGRP(20-27), bombesin, or GRP(1-16). In contrast to the 120 kDa protein, cyclo-SS and RC-160 blocked binding of the radioligands, indicating the 63 kDa protein recognized the modified tetrapeptide sequences ^{2}Phe-D-trp-Lys-Thr[benzyl] and ^{3}Tyr-D-trp-Lys-Val in these analogs. The ability of the 63 kDa protein to influence the binding of GRP to its receptor was investigated with and without added SS. SS blocked the association of GRP with the 63 kDa protein, increasing the amount of GRP bound to its receptor, and thus enhancing its apparent affinity. As anticipated, the association of bombesin (which lacks the tetrapeptide sequence) with the GRP receptor was not affected by SS. Whether the 63 and 120 kDa proteins modify the amount of GRP bound to its receptor **in vivo** remains to be determined.

H 129 EXAMINATION OF THE CELLULAR RESPONSES OF HUMAN PROSTATIC CELLS DERIVED FROM PATIENTS WITH PROSTATIC HYPERPLASIA TO ANDROGENS AND PLATELET DERIVED GROWTH FACTOR. Hirsch, K.S., Gleason, P.E., Eble, J.N., Lamph, W.W., Stamm, N.B., Vlahos, C.J. Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285 [KSH, NBS, CJV], Department of Urology [PEG], and Department of Pathology [JNE], Indiana University Medical Center, Indianapolis, IN 46202, Ligand Pharmaceuticals, Inc. San Diego, CA [WWL]

Androgens are known to play a key role the the growth, differentiation and physiologic function of the prostate and have been implicated as an etiologic factor in the development of prostatic hyperplasia (BPH). Whether these hormones are acting directly or indirectly through the regulation of the expression of growth factors and growth factor receptors has yet to be determined. The presence of a number of polypeptide growth factors in prostatic tissue has led to the speculation that these factors may also play a role in the development of hyperplasia. Histopathologic evaluation of tissue obtained from patients with BPH has revealed that the disease occurs in conjunction with chronic patterns of inflammation. This observation has raised the possibility that platelet-derived growth factor (PDGF), which is released in response to inflammation, may also play a role in the development of the disease. Primary cultures of prostate cells derived from patients who underwent transurethral resection of the prostate (TURP) for the treatment of BPH were established and could be maintained for up to five months. Since the most common form of BPH involves stromal hyperplasia experiments were conducted using prostatic fibroblasts. These cells expressed both androgen and high affinity PDGF receptors. The number of PDGF receptors was found to be inversely related to cell density. Binding of PDGF by its receptor resulted in the activation of a signal transduction pathway which is characterized by receptor autophosphorylation and the activation of phosphtidylinositol-3'-kinase. In addition, exposure of the cells to PDGF resulted in a dose-dependent stimulation of [^{3}H]-thymidine incorporation and cell proliferation. Unexpectedly, the prostatic cells failed to exhibit a mitogenic response to androgenic stimulation. Exposure of the cells to androgens had no effect on the number or affinity of the PDGF receptors or the cellular response to PDGF. Although androgens are believed to be important for the development of BPH their role remains unclear. The results of these studies do suggest that PDGF may be an etiologic factor in the development of prostatic hyperplasia. Attempts are currently underway to examine the the effects of androgens and PDGF on normal human prostate cells.

H 130 Alternative Exon Splicing of Vascular Endothelial Growth Factor RNA Results in Multiple Proteins with Varied Secretion Patterns. K.A. Houck*, N. Ferrara†, and D.W. Leung*. Dept. of *Molecular Biology and †Cardiovascular Research, Genentech, Inc., South San Francisco, CA 94080.

Vascular endothelial growth factor (VEGF) is a direct-acting mitogen apparently specific for endothelial cells and capable of stimulating angiogenesis in vivo. Human VEGF is encoded by a single gene but alternative splicing of its RNA leads to transcripts encoding four different molecular species of VEGF. These forms contain, following signal peptide cleavage, 121 amino acids ($VEGF_{121}$), 165 amino acids ($VEGF_{165}$), 189 amino acids ($VEGF_{189}$), or 206 amino acids ($VEGF_{206}$). The two longer forms of VEGF, $VEGF_{189}$ and $VEGF_{206}$, both contain a 24 amino acid insertion highly enriched in basic amino acids. In contrast to $VEGF_{121}$ and $VEGF_{165}$ which lack this 24 amino acid insertion, the longer forms are not secreted in a freely soluble form into the conditioned medium of human embryonic kidney 293 cells transiently transfected with their cDNAs. The polyanionic compound suramin, which interferes with growth factor/growth factor receptor interactions for VEGF, PDGF, EGF, etc., induced the release of mature $VEGF_{189}$ and $VEGF_{206}$ into conditioned medium of transiently transfected 293 cells. VEGF was released from an unknown extracellular compartment as indicated by the ability to iodinate these molecular species external to the cell. Thus alternative splicing of VEGF RNA can produce transcripts encoding four polypeptides with strikingly different secretion patterns which suggests multiple physiological roles for this family of growth factors.

H 131 EPIDERMAL GROWTH FACTOR ACTION IN THE MOUSE UTERUS INVOLVES THE ESTROGEN RECEPTOR, Diane Ignar-Trowbridge, Karen G. Nelson, Mark C. Bidwell, Sylvia W. Curtis, Todd F. Washburn, Kenneth S. Korach, and John A. McLachlan, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

Epidermal growth factor (EGF) mimics many of the effects of estrogens on the murine female reproductive tract and may partially mediate estrogen-induced growth and differentiation. At present, the mechanism by which the actions of estrogens and EGF converge is unknown. This study addressed the possibility that some of the physiological actions of EGF may involve the estrogen receptor (ER). EGF was administered to ovariectomized mice via slow-release pellets implanted under the kidney capsule. The induction of uterine phosphatidylinositol lipid turnover and DNA synthesis by EGF or diethylstilbestrol (DES), a potent estrogen, was attenuated by the ER antagonist, ICI 164,384. Furthermore, EGF mimicked estrogen-induced modifications of biochemical characteristics of the ER. Estrogen enhances ER affinity for chromatin such that the receptor is retained in the nuclear fraction after tissue homogenization. Similarly, augmented nuclear ER levels were observed after 1.5 hours of EGF treatment as assessed by a [^{3}H] estradiol binding assay and Western blot analysis. Gel retardation studies using a vitellogenin A2 ERE also showed that the nuclear ER extracted from uteri of EGF-treated mice interacted with DNA in a similar manner to ER from DES-treated mice. Finally, EGF treatment induced the formation of an ER form found exclusively in the nucleus after estrogen treatment. These results suggest that EGF may mimic some of the effects of estrogen in the mouse uterus by an interaction between the EGF signaling pathway and the classical estrogen receptor.

H 132 HYDROGEN ION AS A SECOND MESSENGER: INTRACELLULAR ACIDIFICATION ACTIVATES MULTIPLE SIGNALLING EFFECTORS IN SYRIAN HAMSTER EMBRYO CELLS

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Growth factor stimulation of cells involves the use of second messenger systems including calcium/calmodulin, inositol phosphates, cyclic AMP/GMP and phosphate moieties. In research presented here, we have found that hydrogen ions are used by Syrian hamster embryo (SHE) cells as a second messenger. In SHE cells, addition of either epidermal growth factor (EGF) or fetal calf serum (FCS) results in intracellular acidification (in the presence of bicarbonate). Intracellular acidification by itself (without growth factor addition) results in cellular proliferation, immediate early gene transcription, serine/threonine/tyrosine protein phosphorylation, and de novo protein synthesis. Similar events were also observed with growth factor (EGF or FCS) addition. Intracellular calcium levels are not altered in response to intracellular acidification. Finally, intracellular alkalinization resulted in the blunting of EGF-induced proliferation, immediate early gene transcription, and tyrosine phosphorylation. These results indicate that hydrogen ions play an important role in mitogenic signal transduction in SHE cells.

H 133 CHARACTERIZATION OF RECOMBINANT HUMAN HEPATOCYTE GROWTH FACTOR(rhHGF): ACTIVITIES, PROCESSING AND HIGH-AFFINITY RECEPTOR-MEDIATED INTERNALIZATION/DEGRADATION.

Takehisa Ishii, Daiji Naka, Takeshi Shimomura, Yoshiko Yoshiyama Maki Sato, Tadashi Hishida and Hiroto Hara.
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Recombinant human hepatocyte growth factor (rhHGF) was prepared from the culture supernatant of CHO cell transfectant which contained hHGF cDNA. Using rhHGF, biological activities for hepatocyte growth and function, and HGF-receptor interaction were investigated.

1. DNA synthesis of primmary cultured hepatocytes were stimulated by rhHGF in a dose dependent manner. Dose-response curves were identical to native hHGF, and the half-maximal stimulation of DNA synthesis by rhHGF occurred at 20pM, while that of EGF, TGF-α known as hepatocyte stimulators observed at 200-500pM. Furthermore, rhHGF stimulated the production of proteins and the lipid synthesis of hepatocytes.

2. ^{125}I-rhHGF binding assays were performed on hepatocytes and other epithelial cells. Two types of the receptor for rhHGF were detected on these cells; high-affinity($Kd=10^{-11}$M, 10^2-10^3 sites/cells) and low-affinity($Kd=10^{-9}$M, 10^4-10^5 sites/cells) receptors.

3. High-affinity bound ^{125}I-rhHGF was rapidly internalized, degraded and the products were excreted into the culture fluid. The $t_{1/2}$ of rhHGF degradation was estimated as 60-80mim at 37℃. The internalization and degradetion of rhHGF were both temperature dependent.

4. CHO cell transfectants produced two species of rhHGF, i.e., heterodimeric processed form(P-HGF) and immature non-processed form(NP-HGF). Both P- and NP-rhHGF were effective for hepatocyte cell growth. It was suggested that cultured hepatocyte itself cleaved NP-rhHGF to the mature form with the protease.

H 134 MECHANICAL STRAIN INDUCES GROWTH OF VASCULAR SMOOTH MUSCLE (VSM) CELLS THROUGH PRODUCTION OF PARACRINE GROWTH FACTORS. Harlan E. Ives and Qing Mai. Nephrology Division, Cardiovascular Research Institute, University of California, San Francisco, SF, CA 94143

In situ, VSM cells are subjected to continual cyclic deformation. VSM cells grown in rubber bottom plates, when subjected to cyclic stretch (60 cycle/min, 24h), exhibit increased thymidine incorporation and cell number compared to controls. To determine if this is a direct effect of stretch at the single cell level or if it is mediated by secreted factors, mitotic index was measured by thymidine autoradiography over regions of the plates with differing levels of mechanical strain. Control incorporation on unstretched dishes was $10 \pm 1\%$ after 48 h. Strain is maximal (approx. 20%) at the periphery of dishes subjected to stretch. At the periphery, thymidine incorporation was $42 \pm 3\%$ of nuclei (n=10), while at the center, where strain is <3%, incorporation was $27 \pm 2\%$ (n=10; $p<.001$ compared to periphery). The nearly 3 fold increase over control in labeled nuclei at the center of dishes suggests possible secretion of a substance at the periphery which may diffuse to the center. Consistent with this notion, maximal rate of DNA synthesis required 48 h of stretch, compared to 12-18 h for conventional growth factors. Medium from cells that had been stretched 48 h increased thymidine incorporation 25% when added to test VSM cells compared to medium taken from unstretched cells. Lastly, stretch dramatically increased expression of the PDGF A-chain over 24h from 9.6 densitometry units on Northern blots in unstretched controls to 31 units in cells exposed to stretch. No change was observed using a probe for β_2 microglobulin. In conclusion, cyclic stretch is a potent stimulus to growth of VSM; this effect may be mediated by secretion of PDGF-A chain and/or other paracrine growth factors.

H 135 Regulation of glucose transport and the expression of glucose transporter gene by growth factors in mouse 3T3 cells.

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Elevation of a facilitated glucose transport is a well-established feature associated with cell growth and malignant transformation. We have recently shown that a cell cycle-dependent stimulation by serum or growth factors such as PDGF, FGF and PMA of glucose transport in quiescent mouse 3T3 cells is accompanied by a significant increase in gene expression of a brain-type glucose transporter (GLUT1), which is an integral membrane glycoprotein with m.w. of 55 kD. The stimulated increase in glucose transport and the GLUT1 gene expression by serum or one of these growth factors was synergistically enhanced by transforming growth factor-β1 (TGF-β1). The amounts of GLUT1 protein in the TGF-β1-treated 3T3 membranes was shown to be also increased in a synergistic manner. The synergistic stimulation of glucose transport and the GLUT1 gene expression in 3T3 cells peaked at the mid-G1 phase and was completely suppressed by actinomycin D, suggesting that the stimulated glucose transport by these growth factors is mainly due to the enhanced transcription of the GLUT1 gene. A newly synthesized protein was not involved in the stimulation of GLUT1 gene expression, since it was not affected by a protein synthesis inhibitor, cycloheximide. Possible roles of TGF-β1-dependent increase in glucose transport in control of cell growth are discussed.

H 136 ENHANCED EXPRESSION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR AND TRANSFORMING GROWTH FACTOR ALPHA IN HUMAN PANCREATIC CANCER, Murray Korc,* Bysani Chandrasekar,* Michael Kobrin,* Helmut Friess, Markus Buchler, and Hans G. Beger, *Division of Endocrinology and Metabolism, Departments of Medicine and Biological Chemistry, University of California, Irvine, CA, 92717; and Department of Surgery, University of Ulm, Ulm, Germany.
The epidermal growth factor (EGF) receptor is activated by both EGF and transforming growth factor-alpha (TGF-α). However, in cultured human pancreatic cancer cells, TGF-α is a more potent mitogen than EGF. Using immunostaining techniques we now report that the EGF receptor and TGF-α are found in both pancreatic acini and ducts in the normal human pancreas, but are expressed at higher levels in the pancreatic ducts. Northern blot analysis of RNA isolated from human acinar and duct cells indicates that the respective mRNAs are expressed in both cell types, higher levels occurring in the pancreatic ducts. Both proteins are overexpressed in human pancreatic cancer tissues, especially in areas of ductular proliferation and within nests of tumor cells. In these tumors, there is a 3- and 10-fold increase in the mRNA levels encoding the EGF receptor and TGF-α, respectively, by comparison with the normal pancreas. However, amylase mRNA levels are significantly decreased in the tumor samples, indicating that the tumors do not exhibit a generalized increase in gene expression. These findings suggest that TGF-α is synthesized in the normal human pancreas where it may exert autocrine, paracrine, and juxtacrine effects, and that the concomitant overexpression of the EGF receptor and TGF-α in human pancreatic cancer tissues may provide a potent growth advantage to pancreatic cancer cells. Supported by NCI Grant CA-40162.

H 137 GROWTH REGULATION OF NORMAL HUMAN ECTOCERVICAL EPITHELIAL (HEcCE) CELLS BY EPIDERMAL GROWTH FACTOR (EGF) AND TRANSFORMING GROWTH FACTOR-α AND -β(TGF-α AND -β) IN AN OUTGROWTH CULTURE SYSTEM. Tohru Masui, Laboratory of Viral Oncology, Cancer Institute, Toshima-ku, Tokyo170, Japan
We have developed an outgrowth culture system of normal human ectocervical epithelial (HEcCE) cells in order to analyze growth control of normal epithelial tissue. Against our expectation, the presence of serum and high concentrations of calcium (>0.9 mM) promoted growth of the primary outgrowth. However, in consistent with the previous reports, high concentrations of calcium and/or serum inhibited the secondary clonal growth of the cells obtained as an outgrowth in the presence of serum and high calcium. The results suggested uniqueness of growth regulation in the outgrowth culture system and, in the first place, we studied the effects of various growth factors on this system in the presence of high calcium and serum. EGF and TGF-α equally promoted growth in a dose dependent manner up to 10 ng/ml. Since considerable levels of mRNAs for EGF/TGF-α receptors and TGF-α were detected in these cells, TGF-α may serve as an autocrine growth factor in this system. At higher concentrations (>100 pg/ml) TGF-β inhibited growth in a dose dependent manner. Surprisingly, TGF-β at lower concentrations (10-30 pg/ml) promoted the growth of HEcCE cells. These results suggest that the growth regulation of HEcCE cells in an outgrowth may be distinct from that in the conventional serum-free system and have a stronger resemblance to that of wound-healing processes in in vivo. Further studies with this outgrowth culture system will enable a better understanding of the dynamic nature of growth control in epithelial tissue.

H 138 **Analysis of Basic FGF Phosphorylation Using Site-Directed Mutagenesis of Serine and Threonine Residues** *Risë Matsunami, Isabelle Vilgrain, Emelie Amburn and Andrew Baird.* The Whittier Institute for Diabetes and Endocrinology, 9894 Genesee Ave., La Jolla CA 92037

Studies from our laboratory have shown that basic FGF can act as a substrate for phosphorylation by the purified serine/threonine protein kinases, PK-A and PK-C (Proc.Natl.Acad Sci. USA **86**: 3174, 1989) as well as kinases found on the outer cell surface of cultured cells (Mol. Endo. **5**: 1003, 1991) and in purified nuclei of cultured cells. The presence of a phosphorylated FGF indicates that phosphorylation may play a role in how the cell can utilize or respond to FGF. If phosphorylation of bFGF acts as a regulatory mechanism for certain cellular functions, then it would be advantageous to determine the site of phosphorylation on the molecule. This would allow construction of an FGF molecule lacking this site which may then have potential use to effectively turn on or off a response of the cell stimulated by FGF.

In an attempt to determine which serine and threonine residues in bFGF are targeted by these protein kinases, we have mutated the molecule, deleting one or two serine or threonine residues at a time. The biological activity of these altered molecules was then characterized by ability to bind to the FGF receptor, stimulation of cell growth, and ability to stimulate the uptake of tritiated thymidine into nascent DNA by 3T3 fibroblasts. Those molecules that were biologically active were then assayed as substrates for the different purified kinases or for the kinase localized on the surface of SK-hep cells. Thus far we have made single substitutions of all the serines and threonines located between amino acid 50 to 155, and have made several double mutants.

Phosphoamino acid analysis and proteolytic digests were performed on some of the mutant proteins phosphorylated by the different kinases to determine whether the site of phosphorylation in the mutant proteins was the same as that for the wild type molecule. The results indicate that there are alternative sites of phosphorylation in the molecule that are revealed by the deletion of the primary site, and suggest that the growth factor may have more than one site targeted by phosphorylation *in vivo*.

H 139 **ESTROGEN DEPENDENCE OF A BREAST CARCINOMA CELL LINE IS OVERCOME BY TRANSFECTION OF A SECRETED FIBROBLAST GROWTH FACTOR.** **McLeskey, S.W., Honig, S.F., Gelmann, E., Zwiebel, J., Lippman, M.E., and Kern, F.G., Lombardi Cancer Research Center, Georgetown University, Washington, D.C. 20007.** A subset of estrogen receptor positive breast tumors depend on estrogen for growth and are amenable to antiestrogen therapy; such tumors may then become refractory to antiestrogen. This phenomenon has been attributed to outgrowth of a population of tumor cells which express growth factors which stimulate tumor growth in autocrine or paracrine fashion. Fibroblast growth factors (FGFs) are proteins which are mitogenic for cells of mesodermal and neuroectodermal origin. FGFs are also transforming in a number of *in vitro* assays and have been linked to tumorigenicity and metastasis in mouse mammary carcinomas. We have detected mRNA for secreted FGFs in estrogen independent breast carcinoma cell lines but not in estrogen dependent ones, suggesting a role for secreted FGFs in the progression of breast cancer from estrogen dependent to estrogen independent.

To test the hypothesis that FGFs can transform an estrogen dependent breast carcinoma cell line into an estrogen independent one, we have transfected an estrogen dependent cell line, MCF-7, with an expression vector containing sequences for FGF-4 and identified three clonal cell lines (MKS cells) expressing high levels of FGF-4. All the MKS cell lines form large, progressively growing tumors in ovariectomized nude mice which are inhibited by estrogen replacement and stimulated by antiestrogen treatment of the mice. These tumors are metastatic in about 25% of cases. MCF-7 cells and control clones form a few small, nonmetastatic tumors only in ovariectomized mice with estrogen replacement. A histological stain for ß-galactosidase in tumor sections indicates that the MKS cells can support the growth of the parental cell line transduced with a retroviral vector containing sequences for bacterial ß-galactosidase when it is injected together with the MKS cells into the same site. These data support a possible role for a secreted FGF in progression of breast tumors from estrogen dependent to estrogen independent.

H 140 DESIGN AND EXPRESSION OF POTENTIAL PDGF RECEPTOR ANTAGONISTS, Arne Östman, Maria Andersson, Gudrun Bäckström, Ulf Hellman, Bengt Westermark[2] and Carl-Henrik Heldin, Ludwig Inst. for Cancer Research, Box 595, S-751 24 Uppsala, Sweden and [2]Dept. of Pathology, University Hospital, S-751 85 Uppsala, Sweden.

PDGF is a family of disulfide-linked dimeric growth factors. The effects on target cells are mediated by binding to and activating tyrosine kinase receptors. According to the present model for receptor activation the intrinsic tyrosine kinases of the receptors are activated by receptor dimerization induced by the binding of the divalent ligand. This model thus predicts that monovalent ligands would bind to, but not activate, the receptors and be competetive receptor antagonists. To test this prediction we have followed two strategies.

First, the cysteine residues involved in inter-molecular disulfide bridges were identified and cDNAs encoding PDGF A- and B-chains with these cysteine residues changed to serine residues were constructed. These mutants were expressed transiently in COS cells, and analysis by immunoprecipitations and SDS-PAGE revealed that they appear as monomeric forms with retained intramolecular disulfide bridges.

Secondly, a PDGF A-chain derivative, PDGF-0, has been constructed that does not bind to the receptor but forms dimers with wild-type PDGF A-chain. An expression system in which PDGF-A0, but not -AA or -00, is formed has been established.

The functional properties of the monomeric PDGF A- and B-chains and of PDGF-A0, are now being examined.

H 141 INITIAL CHARACTERIZATION OF LOW MOLECULAR WEIGHT FORM OF TRANSFORMING GROWTH FACTOR TYPE E, Pamela G. Parnell*, Richard Harkins+, Bobbie Carter* and Jaroslava Halper*, *Department of Pathology, College of Veterinary Medicine, Athens, GA 30602 and +Berlex Biosciences, Alameda CA 94501

Transforming growth factor type e (TGFe) acts as a mitogen for epithelial and fibroblastic cells in monolayer and soft agar. Partial amino acid sequence of H.M.W. of TGFe (M, 25,000) showed no similarity to other growth factors. In this study we report the isolation of a L.M.W. form of TGFe. This 20,000 M, form coelutes with the H.M.W. form during its initial purification steps from bovine kidney; however, it can be separated from H.M.W. TGFe by high pressure electrophoresis chromatography (HPEC). The biological activity of L.M.W. TGFe is lower than that of the H.M.W. The partial amino acid sequence of L.M.W. TGFe appears to be unique and different from that of H.M.W. TGFe. Deglycosylation of H.M.W. TGFe by N-glycanase yielded a 20,000 dalton protein with partially retained biological activity. No further deglycosylation of TGFe was noted upon subsequent treatment with neuraminidase and O-glycanase. These data indicate that complex and high mannose N-linked carbohydrate is bound to the protein core. At the present time we do not know whether L.M.W. TGFe represents a proteolytic product of H.M.W. TGFe, differentially deglycosylated form of TGFe or an unrelated protein.

H 142 A 41 kDA TRANSFERRIN RELATED MOLECULE ACTS AS AN AUTOCRINE GROWTH FACTOR FOR HL-60 CELLS. Petro E. Petrides [1,2] and Klaus H. Dittmann [1]

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The human promyelocytic cell line HL-60 survives and grows in chemically defined medium only in the presence of exogenous transferrin and of supraphysiological concentrations of insulin [Collins, Blood 70: 1233 (1987)] or physiological amounts of IGF-I, resp. When medium conditioned by HL-60 cells in the absence of insulin and transferrin is collected and concentrated it does promote the growth of HL-60 cells grown in chemically defined medium in the presence of insulin, but only in the absence of exogenous transferrin. This indicates that HL-60 cells produce an autostimulatory survival or growth factor. Since the stimulatory effect of HL-60 conditioned medium is only observed in the absence of exogenous transferrin we have assayed HL-60 cells for the production of transferrin and found that they produce polypeptides which react with transferrin antibodies. ^{35}S-methionine labelling, immunoprecipitation and subsequent separation by SDS-gel electrophoresis reveals the presence of a major transferrin related 41 ± 2 kDa species released by HL-60 cells. In contrast, cell lysates contain several other immunoprecipitable species in addition to this molecule. Physiological levels of iron salts completely abolish the requirement of exogenous transferrin which indicates that the endogenous transferrin related polypeptides in the presence of exogenous inorganic iron salts are sufficient for the proliferation of HL-60 cells provided insulin or related growth factors are present. The addition of transferrin receptor antibodies inhibits the stimulatory action of the endogenous transferrin related activity. We conclude that HL-60 cells produce transferrin related molecules (leukemotransferrins) for autostimulation.

Supported by grants from the Deutsche Forschungsgemeinschaft and GSF.

H 143 TRANSLATIONAL CONTROL OF BASIC FIBROBLAST GROWTH FACTOR EXPRESSION by A.-C. Prats[1], S. Vagner[1], H. Prats[2] and F. Amalric[1]. Centre de Recherche de Biochimie et Génétique Cellulaires du CNRS[1]and Laboratoire d'Endocrinologie Expérimentale[2], Toulouse, France.

The basic fibroblast growth factor (bFGF) is a multifunctional cytokin able to stimulate cell proliferation, migration and differenciation, which also shows oncogenic features. Four forms of bFGF are synthesized from the same mRNA, using alternative translation initiation sites. Three CUG initiation codons are used for translation of the high molecular weight forms (22, 22.5 and 24 kDa, respectively) whereas an AUG codon allows translation initiation of the low molecular weight form of bFGF (18 kDa). We have recently shown that the small AUG-initiated form localizes in the cell cytoplasm and is able to induce cell transformation, while the large CUG-initiated forms are localized in the cell nucleus and generate cell immortalization. These observations indicate that the process of alternative translation initiation can regulate the expression of proteins having not only different subcellular localization but also distinct functions, involved in the control of cell growth and differenciation. The roles of the 5' untranslated and of the alternatively translated regions in the control of alternative translation has been investigated *in vitro* and *in vivo*. Site directed mutagenesis has allowed us to map within the bFGF mRNA cis-acting regions able to modulate alternative translation initiation of the different forms of bFGF and allowing cap-independent translation. These regulatory elements presumably correspond to secondary or tertiary RNA structures which could be targets for cell specific translation regulating factors.

H 144 ASSOCIATED PHENOTYPES OF THE bFGF NATIVE FORMS WHEN EXPRESSED IN ENDOTHELIAL AND VARIOUS TRANSFORMED CELLS.
By Hervé Prats, Béatrix Bugler*, Bettina Couderc, Veronique Patry*, Catherine Zanibellato, Arlette Maret, Michel Weber and François Amalric*.
Laboratoire d'Endocrinologie Expérimentale, CHU Rangueil, Bât L3 , 31054 Toulouse, FRANCE . and (*) CRBGC du CNRS, 118 route de Narbonne, Toulouse, FRANCE

The subcellular localization of the different forms of bFGF is directed by alternative used of initiation codons. The AUG initiated form (bFGF 155aa) is predominantly cytoplasmic while the two CUG initiated forms (210 and 195aa) are nuclear.
In a way to understand the biological significance of such a differential localization we have constructed retroviruses expressing one, two or the three natives bFGFs forms. Constitutive expression of these constructs in bovine aortic endothelial cells (ABAE) induced different phenotypes caracterised by their oncogenic behaviour and by their responses to various stimulus, particulary for exogenous bFGF. In a way to understand the relationship between the cell expression of bFGF and its sensibility to the factor, the affinity and the number of bFGF receptors was investigated. In an other hand each cell line were transfected by the gene encoding the FGF-R2 receptor and we have analyzed the associated phenotypes.
We also expressed bFGF cDNA in various established cell lines including HeLa, neuroblastoma and carcinoma cells. Our results indicate that constitutive expression of bFGF deeply modify the behaviour of these different cell lines. The high molecular forms of bFGF seem to be involved in the regulation of gene expression while the AUG initiated form is more involved in oncogenic processes.

H 145 METALLOTHIONEIN-REGULATED TGF-β_1 SECRETION IN MALIGNANT FIBROSARCOMAS STIMULATES LOCOMOTION BY REGULATING A NOVEL HYALURONIC ACID (HA) RECEPTOR. Shanti K. Samuel, Robert A.R. Hurta, Eva A. Turley and Arnold H. Greenberg. Manitoba Institute of Cell Biology, 100 Olivia Street, Winnipeg, Manitoba, R3E 0V9 Canada.
A *ras-transformed fibrosarcoma was transfected with a* plasmid (pPK9A) containing the complete coding region of porcine TGF-β_1 under the control of a metallothionein promoter. The TGF-β_1 cDNA was mutated replacing cysteines 223 and 225 with serines which allowed secretion of bioactive TGF-β_1. A clone (17.18) was selected in which a 6-fold increase in TGF-β_1 mRNA was found following zinc-sulfate treatment. Coincident with increased TGF-β_1 expression, these tumor cells became progressively more motile. To further characterize the locomotory properties of these cells, TGF-β_1 regulation of the expression of a newly characterized 58 kDa HA receptor termed RHAMM was investigated. This protein has previously been shown to regulate the locomotion of *ras*-transformed and embryonic cells. In clone 17.18, mRNA levels as detected by Northern blot analysis, showed elevated levels of RHAMM 24 hours after the TGF-β_1 gene was induced. Preliminary observations with a highly malignant *ras*-transformed cell (C3) revealed that in response to exogenously added TGF-β_1 (10 ng/ml), an elevation in RHAMM mRNA expression occurs as early as 1 hour post TGF-β_1 exposure. Furthermore, the importance of RHAMM to TGF-β_1 induced locomotion was demonstrated by the ability of monoclonal and polyclonal antibodies specific to RHAMM to completely block locomotion induced by TGF-β_1, without altering the motility of unstimulated cells. (Supported by the NCI of Canada).

H 146 REGULATION OF GROWTH FACTOR PRODUCTION CORRELATES IN LACRIMAL GLAND WITH WOUND RESPONSES FROM THE EYE, Hilary W. Thompson and Roger W. Beuerman, LSU Eye Center and Laboratory of Molecular Biology of the Ocular Surface, New Orleans, LA 70112
The lacrimal gland produces a variety of enzymes, antimicrobial and growth factors that are secreted or released into tears and which may have a regulatory effect on cells of the front of the eye, the cornea and conjunctiva. In NZ albino rabbits, EGF and EGF receptor (EGFR) messenger RNAs, and the EGF protein changes were determined in lacrimal gland extracts, and in tears, respectively. To initiate the response,a shallow circular 6 mm diameter wound was made in the central cornea. Such wounds have been used in our laboratory as a bioassay of the re-epithelialization process and are highly regular in their time course of closure. EGF mRNA is constitutively produced in the lacrimal gland and the released EGF may have a role in the normal turnover of corneal epithelium. At 2 hours after a corneal wound the 4.8 kb mRNA for the growth factor precursor is increased 2 fold above the control level. At 12 hours an increase of EGF protein, mainly the 130 kd precursor, is seen in lacrimal gland extracts. Increased amount of the precursor appears in tears at 24 hours. The EGF receptor has two transcript sizes in lacrimal gland. A 5.5 kb full sized transcript, and a truncated 2.2 kb transcript, similar to transcripts which produce the outer membrane domain EGF binding part of the receptor. This is believed to be secreted as an extracellular EGF binding protein. The truncated transcript shows a significant increase in expression at 1 to 2 hours after wounding. These molecular events are preceded by increases of the primary response genes c-fos, c-myc and c-jun at 15 minutes following the corneal wound. The roles and interrelationships of these events and the regulation of EGF production and release suggest the operation of a neural connection activated by the wound process.

H 147 DIFFERENTIAL EFFECTS OF PDGF ISOFORMS ON *PROLIFERATION OF NORMAL RAT KIDNEY CELLS*, Everardus J.J. van Zoelen, Walter van Rotterdam, Carl-Henrik Heldin, Department of Cell Biology, University of Nijmegen, The Netherlands, and Ludwig Institute, Uppsala, Sweden
The effects of the PDGF isoforms AA, AB and BB have been studied on the proliferation of normal rat kidney fibroblasts which contain both type α and type β PDGF receptors. On monolayer cells made quiescent by serum deprivation, PDGF-AA is a relatively poor mitogen compared to PDGF-AB and PDGF-BB. When these cells are made density-arrested following continuous incubation with epidermal growth factor, however, they can be restimulated to proliferate by all three PDGF isoforms with similar activity when added at sufficiently high concentration, resulting in phenotypic cellular transformation. Binding of radiolabelled isoforms to confluent NRK monolayers obeys the predictions of an induced receptor dimerisation model, and increases in the order AA<AB<BB. Upon preincubation of the cells with PDGF-AA, the dose-response curve for mitogenic activity of PDGF-AB is shifted to higher concentrations, indicating that PDGF-AA can partly antagonize the growth stimulating activity of PDGF-AB, as has also been ob- served in ligand binding studies. PDGF-AB is highly active in inducing anchorage-independent proliferation of NRK cells, but in all such assays PDGF-AA is at least as potent as PDGF-BB. Intriguingly, PDGF-BB is almost devoid of activity in inducing soft agar growth of these cells, in contrast to PDGF-AA. These results show that the relative potency of the three PDGF isoforms to stimulate proliferation of NRK cells is different for quiescent cells in monolayer, density-arrested cells and anchorage-independent cells. Moreover it is shown that the biological activity of PDGFs can be impaired by the additional presence of other isoforms.

H 148 PHOSPHORYLATION OF BASIC FGF IN THE NUCLEI OF SK-HEP CELLS

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In order to better understand the mechanism of basic FGF action, previous studies from our laboratory have focused on its potential regulation by phosphorylation. For example, we have shown that basic FGF is a substrate for phosphorylation by purified PK-A and PK-C and that it is synthesized as a phosphoprotein by cells in culture (Proc.Natl.Acad. Sci.USA **86**:3174, 1989). The phosphorylation of basic FGF is also modified by heparin and selected proteins of the extracellular matrix (J.Cell Biol. **109**:3105, 1989) suggesting a potential regulatory role for an ectokinase that phosphorylates basic FGF on the outer cell surface of target cells (Mol.Endo. **5**:1003, 1991). Because recent studies have now localized basic FGF in the nucleus (Growth Factors **4**:265-275), we have extended these observations in an attempt to identify whether basic FGF is phosphorylated in this compartment as well.

SK-Hep cells are derived from a human hepatoma cell line which synthetize relatively large amounts of basic FGF and possess over 5000 high affinity receptors/cells. When a homogenate of SK-Hep cells is fractionated by differential centrifugation to separate membranes, cytosol and nuclei, and each is incubated with [γ-^{32}P]-ATP and basic FGF, both membrane and nuclear fractions contain a kinase capable of phosphorylating the growth factor. A further analysis shows that this latter reaction is time and concentration dependent and targets serine residues as determined by phosphoamino acid analyses. The reaction is unaffected by the addition of calcium and/or phospholipids but is effectively inhibited by heparin (1 ug/ml) and spermidine (2 mM). Chromatographic analyses indicate that the nuclear FGF kinase is not casein kinase II (CK-II).

These data establish the existence of a kinase capable of phosphorylating basic FGF in the nuclei of SK-Hep cells. It appears distinct from the well characterized kinases (PK-A, PK-C, CK-II) that are known to phosphorylate basic FGF. Because the kinase activity is also found in the nuclei of other cell lines (3T3 cells, bladder carcinoma cells), we are using a number of mutated FGFs to determine whether or not this reaction is linked to the cell cycle or to basic FGF's ability to stimulate cell proliferation and differentiation.

Receptors and Phosphatases

H 200 SIGNALLING MECHANISMS THROUGH CD5

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In this report we describe a novel pathway of human T cell activation and proliferation involving the CD5 surface antigen. The CD5-specific Cris-1 mAb induces by itself monocyte-dependent proliferation of PBMC. Among a panel of CD5-specific mAbs (Leu-1, OKT-1, LO-CD5a, F101-1C5, F145-GF3), only the F145-GF3 mAb shared this property with Cris-1. The analysis of he biochemical pathway involved in this activation showed the lack of detectable hydrolysis of inositol phosphates or early increments in $[Ca^{+2}]_i$ after triggering cells with the mitogenic CD5 mAb. However stimulation with CD5 induces activation of PKC, as measured by phosphorylation of a specific peptide substrate (peptide GS), that can be inhibited by a pseudosubstrate peptide inhibitor. Stimulation with CD5 mAbs induces also tyrosine kinase activity, with a substrate pattern that differs from that induced after triggering lymphocytes through the TCR/CD3 complex. In fact, under the adequate detergent conditions CD5 coimmunoprecipitates a kinase activity different to that coimmunoprecipitated by CD3 and CD4. On the other hand the IL2/IL2R pathway seems involved in the CD5-mediated proliferation of PBMC, for anti-IL2R specific mAb inhibits CD5-induced proliferation, and stimulation with mitogenic CD5 mAbs induces production of IL-2 and expression of IL2R α and β chains. Therefore, the triggering of the CD5 antigen can induce IL-2 and monocyte-dependent human T cell proliferation by a biochemical pathway that differs, at least in the first stages, from the one that mediates TCR/CD3 complex-induced T cell activation.

H 201 THE CD45 PHOSPHOTYROSINE PHOSPHATASE REGULATES T CELL ANTIGEN RECEPTOR COMPLEX MEDIATED DIACYLGLYCEROL PRODUCTION AND PROTEIN KINASE C ACTIVATION IN HUMAN T CELLS.

Denis Alexander[1], Mark Biffen[1], Lindsey Goff[2] and Emer Shivnan[1].
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Antigen recognition by the T cell antigen receptor complex (TCR) or the binding of agonistic antibodies against the CD3 antigen results in increased inositol phosphate (IP) and diacylglycerol (DAG) production, and a release of Ca^{2+} from intracellular stores, with a consequent activation of protein kinase C (PKC). A rise in Ca^{2+} and increased PKC activity have been implicated in the mitogenic pathways that regulate expression of the IL2 and IL2R genes. We have shown that CD3 mAb's do not induce IP or DAG production, nor PKC activation, in CD45$^-$ HPB-ALL T leukemia cells. Transfection of CD45 cDNA into CD45$^-$ cells restored coupling of the TCR to IP and DAG production, as well as to PKC activation. However, CD3-induced IP production in CD45$^+$ cells was only 20% above control levels, compared to a 5-fold increase in CD3-induced DAG levels, suggesting that DAG was derived from lipids other than phosphoinositides. These findings point to a positive role for cell-surface expressed CD45 molecules in regulating TCR-coupling to DAG production and PKC activation.

H 202 Expression of functional fibroblast growth factor receptors in several human leukemia cell lines

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With the use of the PCR cloning method we have discovered in the K562 leukemia cells several novel tyrosine kinases, two of which belong to the fibroblast growth factor receptor family (FGFR-3 and FGFR-4; Partanen et al., 1990; 1991). We have therefore analysed the expression of four different FGFRs, including the two novel genes in human leukemia cell lines in culture. Our results show that at least FGFR-1 (*flg*), FGFR-3 and FGFR-4 mRNAs are expressed in several leukemia cell lines at levels similar to those found in solid tumor cell lines. Radioactive ligand crosslinking experiments indicated that at least the K562 cells have cell surface receptors for aFGF. In addition, growth factors appear to regulate of FGF receptors in these leukemia cells. Small amounts of FGFR-4 mRNA were seen in ribonuclease protection of samples from both normal and regenerating bone marrow. Analysis of possible expression FGF receptors in normal hematopoietic cells is underway.

H 203 ANTISENSE OLIGONUCLEOTIDES USED TO STUDY CSF-1 SIGNALLING

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The aim is to identify components essential for signal transduction by the CSF-1 receptor, using antisense oligonucleotides to inhibit expression of potential signalling molecules. Two cell lines are being used for the antisense experiments, NIH 3T3 transfectants expressing the human CSF-1 receptor (NIH/CSF-1R) and BAC1.2F5 cells, the CSF-1-dependent macrophage line. Investigation of oligonucleotide uptake and stability with NIH 3T3s showed that unmodified oligos were degraded rapidly both in cells and medium. In an attempt to reduce degradation, we tested a c-myc phosphorothioate, antisense oligo. This oligo was stable for 24 hrs after uptake and blocked proliferation of NIH 3T3s completely. Uptake and stability of the c- myc oligo by BAC1.2F5 cells are being tested at present.

After these preliminary experiments to check the antisense method, we are using the approach now to investigate the role of protein kinase C (PKC) in CSF-1 signalling. Previous reports on this question conflict somewhat. Activation of PKC with phorbol ester has been shown to result in inactivation of CSF-1R by proteolytic cleavage. More recently, it was reported that CSF-1 stimulates phosphatidylcholine hydrolysis and thus activates PKC in human blood monocytes. Our initial antisense experiment is to try and inhibit expression of PKC-α, assayed by immunofluorescence, in the NIH/CSF-1R transfectants, then test whether this blocks CSF-1-induced proliferation. The potential advantages of the PKC antisense, over phorbol ester down-regulation of PKC, are PKC subtype specifity and the ability to assay cell proliferation in the presence of oligos.

H 204 THE α_{1B} ADRENERGIC RECEPTOR: Structural Domains and Effects of Constitutive Activation on Mitogenesis and Tumorigenicity.

Lee F. Allen, Michael A. Kjelsberg, Marc G. Caron, Robert J. Lefkowitz, and Susanna Cotecchia, Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC 27710.

Elucidation of the primary structure of the adrenergic receptors (ADRs) and other G-protein-coupled receptors has identified several conserved structural features, i.e. 7 putative hydrophobic transmembrane spanning domains joined by three intra- and extra-cellular loops. Studies with chimeric receptors have demonstrated that the third intracellular loop (3i) contains structural determinants for the specificity of receptor/G-protein coupling, which ultimately determine the physiologic response to agonist-induced receptor activation.

To define the key determinants for the selectivity of receptor coupling to phospholipase C (PLC), probably mediated through G_q, a series of chimeric receptors was constructed involving substitution of selected regions of the α_{1B}-ADR 3i into the β_2-ADR. These chimeric receptors bind ligands with the specificity of the β_2-ADR, but are coupled to the effector system of the wild type α_1-ADR, i.e. PLC-mediated phosphoinositide (PI) hydrolysis. Through selective mutagenesis, a sequence of 27 amino acid residues in the N-terminus of the 3i has been identified, that appears to contain the minimal structural determinants required for coupling the receptor to PLC.

The C-terminus of the 3i also appears to have important effects on G-protein coupling. Mutagenesis studies on the α_{1B}-ADR demonstrated the ability of a point mutation at a single site (Ala^{293}) to render this receptor constitutively active, resulting in the stimulation of PI hydrolysis in the absence of agonist-induced receptor activation. In addition, this activating mutation was found to increase the binding affinity of norepinephrine at the receptor. Substitution of all 19 potential amino acids at this site resulted in constitutive activity and enhanced agonist binding affinity, suggesting that this structural domain may play a key role in constraining the normal physiologic functioning of the receptor.

Recent studies on the α_{1B}-ADR have demonstrated the potential of this receptor to function as a growth factor and proto-oncogene with agonist-induced mitogenesis, focus formation *in vitro* and tumorigenesis *in vivo*. Mutational alteration of the α_{1B}-ADR, which induced constitutive activity, also appeared to activate this proto-oncogene. "Oncomutant" expressing cell lines demonstrated a more aggressive transformed phenotype, and exhibited agonist-independent focus formation and maximal growth rates.

These findings identify structural domains in the N-terminus of the 3i of the α_{1B}-ADR as determinants for the specificity of receptor coupling to PLC, and implicate a key region in the C-terminus of this loop in modulating the functional consequence of receptor/G-protein coupling by inducing constitutive activity. Mutational alteration of this region of the receptor may prove to be a general mechanism for inducing constitutive activity, and subverting these or other structurally related receptors *in vivo*, leading to enhanced mitogenesis and human disease, e.g. neoplasia and atherosclerosis.

H 205 INTERNALIZATION AND DOWNREGULATION OF A GROWTH HORMONE RECEPTOR MUTANT DEFECTIVE IN SIGNAL TRANSDUCTION.

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Growth hormone binds specifically to receptors on pancreatic β- cell stimulating proliferation and insulin production. Similar effects have been demonstrated in RIN-5AH, a rat insulinoma cell line, expressing low levels of specific receptor for GH. RIN-5AH transfected with the full length rat hepatic Growth hormone receptor ($GH\text{-}R_{1\text{-}638}$) exibits an augmented GH-response. To determine the GH-R structural requirement for ligand-dependent endocytosis, we have engineered, by site directed mutagenesis, two COOH terminally truncated GH receptors retaining binding activity. $GH\text{-}R_{1\text{-}294}$ retains only 5 amino acids after the transmembrane domain and $GH\text{-}R_{1\text{-}454}$ retains 165 amino acids after the transmembrane domain. Wild type and mutant receptors have been expressed in RIN-5AH under the transcriptional control of the human metallothionein promoter. All clones exibited increased binding with similar affinity. None of the GH-truncated receptors was able to transmit the signal for GH-stimulated insulin production. Analysis of GH-uptake mediated by a single cohort of receptors was performed at 37°C after preincubation with ^{125}I-GH at 4°C for 3 h. As determined by acid wash stripping of surface bound ^{125}I-GH the parent cell line and the clones expressing $GH\text{-}R_{1\text{-}638}$ and $GH\text{-}R_{1\text{-}454}$ internalize 60-70% of the specific cell associated ^{125}I-GH after 30 min.at 37°C. After 4 h 40-60% of the total counts were found in the medium as trichloroacetic acid(TCA) soluble counts, indicating efficent GH degradation. In RIN-5AH cells expressing $GH\text{-}R_{1\text{-}294}$, the internalizaton was greatly impaired: after 30 min. 20- 30% ot the ligand was detectable in the intracellular compartment and after 4 h 20-30% of the total counts were found in the medium as TCA soluble counts. Pretreatment of cells expressing wild type and mutant $GH\text{-}R_{1\text{-}454}$ with GH resulted in a rapid downregulation of GH surface receptors while $GH\text{-}R_{1\text{-}294}$ did not show any. We conclude that ligand degradation and receptor downregulation are coupled to initial receptor binding by internalization process and that domains between amino acids 294 and 454 of the GH-R are required for efficent ligand dependent endocytosis.

H 206 FLOW CYTOMETRIC DETERMINATION OF EGF CELL SURFACE RECEPTORS, R.W. Beuerman, J.G. Lopez, J.S. Malter, M.S. Insler, H.W.Thompson, LSU Eye Center Laboratory for the Molecular Biology of the Ocular Surface, LSU School of Medicine, New Orleans, LA 70112

Epidermal growth factor (EGF), a mitogen for many mammalian cells, binds to the extra-cellular domain of a receptor that has an intracellular site for tyrosine phosphorylation. Assays for the EGF-receptor (EGFR) have generally used radio-labelled iodine. These methods have several drawbacks: they are time-consuming, labor-intensive and require a labeled compound.

In the present method, the receptor is labeled with a mono-clonal antibody, a fluorochrome is attached to a secondary complex. If the staining reaction is stoichiometric then by appropriate selection of optical filters and calibration procedures quantitation can be achieved. We have used r-phycoerythrin conjugated to strepavidin and the system calibrated with a Quantum phycoerythrin microbead kit with molecules of equivalent soluble fluorochrome (MESF) ranging from 10*4 to 5 x 10*5. Conversion of MESF to the number of bound antibody molecules/cell considered intermolecular quenching. Nine cell ocular cell lines were examined along with NIH/3T3 and A431 cells. The maximum number of EGFRs was found for A431 cells (>5 x 10*5), while NIH/3T3 cells revealed from 2200_+302 to 800-+302 depending on contact inhibition. Ocular cells all had less than 10*5 EGFRs/cell. In summary, this method allows rapid quantitation of EGFRs, present work is extending this analysis to other growth factor receptors. (Partially supported by USPHS grants EY07608,EY02377 and EY04074)

H 207 OVEREXPRESSION OF c-erbB-2 IN NORMAL AND IMMORTAL HUMAN MAMMARY EPITHELIAL CELLS, Shelley B. Blam[1], Martha R. Stampfer[2], [1]Berlex Biosciences, Department of Cell Biology and Immunology, Alameda, CA 94501; [2]Lawrence Berkeley Laboratory, Division of Cell and Molecular Biology, Berkeley, CA 94720.

The protooncogene c-erbB-2 encodes a putative growth factor receptor-like protein similar in structure to the epidermal growth factor (EGF) receptor. Amplification and over-expression of this gene have been described in a variety of human adenocarcinomas. This has been particularly well-documented in breast cancers where amplification of c-erbB-2 occurs in 10-33% of these tumors. Recent work has suggested that overexpression of the c-erbB-2 protooncogene may play a role in the aggressive clinical behavior of some breast tumors. The mechanism(s) by which c-erbB-2 over-expression contributes to increased proliferative potential remains to be determined. In order to investigate the oncogenic potential of this gene, expression vectors bearing the erbB-2 cDNA were constructed and introduced into both normal diploid and immortal pseudodiploid human mammary epithelial cells. Overexpression of erbB-2 protein was achieved in several clonally-derived populations. The data indicate that overexpression of c-erbB-2 is not sufficient to affect the growth properties of the normal cells, but induced anchorage-independent growth in the immortal derivatives. We will describe data addressing the functionality of the transfected erbB-2 protein as well as differentiation-associated properties of the transfectants.

H 208 MECHANISM OF ACTIVATION AND FUNCTIONAL PROPERTIES OF THE HUMAN C-*KIT* PRODUCT, P. Blume-Jensen, L. Rönnstrand, L. Claesson-Welsh, A. Siegbahn [1], K. M. Zsebo [2], B. Westermark [1] and C.-H. Heldin, Ludwig Institute for Cancer Research, Box 595, Biomedical Center, S-751 23 Uppsala, Sweden, [1] University Hospital, S-751 85 Uppsala, Sweden, [2] AMGEN Inc., AMGEN Center, Thousand Oaks, CA 91320, USA.

The proto-oncogene c-*kit* is allelic with the murine *white spotting* (*W*) locus and encodes a transmembrane tyrosine kinase receptor that is structurally related to the receptors for platelet-derived growth factor (PDGF) and colony stimulating factor-1 (CSF-1). The c-*kit* product and its ligand stem cell factor (SCF), are important for three major developmental processes, namely melanogenesis, gametogenesis and haematopoiesis. We have transfected the wild type human c-*kit* cDNA into porcine aortic endothelial cells, and examined its mechanism of activation and some biological properties after stimulation with soluble SCF. The receptor was downregulated and transmitted a mitogenic signal in response to stimulation with SCF. We further found that SCF induces dimerization of the c-*kit* product in intact cells, and dimerization of the receptor is correlated with activation of its kinase. Interestingly, soluble SCF is a potent chemotactic agent for the c-*kit* expressing cells, and activation of the c-*kit* product by SCF induces circular actin reorganization similar to that mediated by the PDGF β-receptor in response to PDGF-BB. We have initiated studies on phosphotyrosylated substrates for the c-*kit* product, and some potential novel substrates have been identified, of which some are shared with the PDGF β-receptor and others are unique.

H 209 IDENTIFICATION OF SPECIFIC INHIBITORS OF EGFR AND SRC TYROSINE KINASES. George B. Boder, Teresa Burke, Lori Eichelberger and Jill A. Panetta. The Lilly Research Laboratories, Indianapolis, IN 46285. Studies on the EGFR and src kinases have provided valuable insights into the causal role of growth factors and their receptors in tumorigenesis. Overexpression of the EGFR gene and src has been found in various human tumors. We have developed comparative sensitive tyrosine kinase assays that have detected compounds with IC50's with lower than uM concentrations. Membranes were purified from A-431 human epidermoid cells resulted in increased sensitivity by reduction of ^{32}P phosphorylation of endogenous proteins. Several different synthetic and natural substrates were compared. The synthetic ploymer (Glu, Ala, Tyr) 6:3:1 generated maximum phosphorylation. Specificity of tyrosine phosphorylation was confirmed by slot-blot assays using anti-phosphotyrosine antibodies. Recombinant src expressed in the Baculovirus system was used to develop an ELISA based assay. Several compounds have been identified that are selective for either src or EGF tyrosine kinase.

H 210 SITE-SPECIFIC MUTAGENESIS OF THE EXTRACELLULAR DOMAIN OF THE HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR,
Pamela M. Brown, Mireille Caron and Maureen D. O'Connor-McCourt, Biotechnology Research Institute, National Research Council Canada, Montreal, Quebec, Canada H4P2R2
Oligonucleotide-directed site-specific mutagenesis of the extracellular domain of the human epidermal growth factor receptor (EGFR-ED) was carried out in order to better understand the mechanisms by which multiple growth factors can bind to the same receptor yet elicit differential responses. The mutated EGFR-ED thus produced was expressed utilizing the baculovirus expression system. Functional analysis of the mutated receptor involved affinity cross-link labeling and equilibrium binding studies with both EGF and TGF-alpha. Further characterization of the mutated EGFR-ED involved its interaction with various anti-EGF receptor monoclonal antibodies. All studies were carried out in parallel with a nonmutated form of the EGFR-ED produced in the same baculovirus expression system in an identical manner. The results of these comparative studies demonstrate that it is possible to differentially affect binding of one ligand (TGF-alpha) while not affecting binding of a second ligand (EGF). Thus, EGF and TGF-alpha appear to interact differently with the EGF receptor which presumably could result in their different biological activities.

H 211 THE PROTEIN TYROSINE KINASES p56^{lck} AND p59^{fyn} INTERACT WITH DIFFERENT CELL SURFACE RECEPTORS
IMPORTANT IN T CELL ACTIVATION, Paul Burn, Kurt Amrein, Martin Gassmann, Department of Biology, PRT, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland
The T lymphocyte cell surface receptors CD4 and CD8, and the T cell receptor (TCR)-CD3 complex, are involved in activating several signal transduction pathways via coupling mechanisms that are poorly understood. One of the earliest events that follows the triggering of T lymphocytes via these receptors is a rapid tyrosine phosphorylation of several intracellular substrates. In order to elucidate the molecular mechanisms involved in T cell signal transduction and activation it is crucial to identify the protein tyrosine kinase(s) (PTKs) which mediate(s) these early signalling events. Since neither CD4 nor CD8, nor the subunits of the TCR-CD3 complex, exhibit any recognizable kinase domains, it is likely that the receptors are associated with nonreceptor PTKs. p56^{lck} and p59^{fyn} are two nonreceptor, *src*-like, cell membrane-associated PTKs expressed in T lyphocytes. In order to study possible physiologically relevant associations of CD4 or CD8 or the TCR-CD3 complex with p56^{lck} or p59^{fyn}, the receptors on the surface of intact human T lymphocytes were collected into caps by crosslinking with specific antibodies, and the capped cells were examined by double immunofluorescence techniques to determine whether either of the PTKs were co-collected with the corresponding cell surface receptor caps. The capping of the different receptors resulted in a co-distribution of p59^{fyn} with the TCR-CD3 caps, and a co-distribution of p56^{lck} with the CD4 or CD8 receptor caps. These results provide direct evidence for a specific and physiologically relevant association of p59^{fyn} with the TCR-CD3 complex and of p56^{lck} with CD4 or CD8 in functional T lymphocytes. Our results suggest that in T lymphocytes multiple PTKs are involved in receptor-associated signal transduction pathways. Currently we investigate what the relationship is between the different pathways and how the differnt PTKs are regulated through differnt cell surface receptors.

H 212 HER-2/*neu* MEDIATED TRANSFORMATION AND TUMORIGENESIS INDEPENDENT OF THE EPIDERMAL GROWTH FACTOR RECEPTOR Victoria R. Chazin and Dennis J. Slamon. Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90024

Amplification of the HER-2/*neu* gene and overexpression of the p185^{HER-2} gene product is found in approximately one-third of primary human breast and ovarian cancers and is associated with a poor clinical outcome of early relapse and death. The HER-2/*neu* gene encodes a cell-surface growth-factor receptor with intrinsic tyrosine kinase activity. The ability of wild type human HER-2/*neu* overexpression to transform cells has been shown, demonstrating that HER-2/*neu* can act as a potent oncogene when overexpressed. Current data suggests that the mechanism by which HER-2/*neu* mediates transformation may involve an interaction between HER-2/*neu* and the EGF receptor. To test whether overexpression of normal human HER-2/*neu* can transform cells independently of the EGF receptor, we have introduced multiple copies of HER-2/*neu* into NR6 cells (a Swiss 3T3 derivative cell line which lacks any EGF receptor), and have performed assays for both transformation and tumorigenicity. Engineered NR6 cells that express high levels of HER-2/*neu* display a highly transformed phenotype as compared to control cells. These HER-2/*neu* overexpressing cells form colonies in soft agar and extremely aggressive tumors in nude mice, whereas control cells do not. Utilizing a monoclonal antibody made to the extracellular domain of the HER-2/*neu* receptor, we are able to inhibit the proliferation of the overexpressing cells *in vitro* as well as tumor growth *in vivo*. This study provides clear evidence that HER-2/*neu* mediated transformation can be achieved independently of the EGF receptor.

H 213 PROTEIN KINASE C βI AND γ, BUT NOT α OR ε, DIRECTLY REGULATE INSULIN RECEPTOR PHOSPHORYLATION. Janice E. Chin, Jeremy M. Tavare*, and Richard A. Roth. Department of Pharmacology, Stanford University, Stanford, CA 94305. *Department of Biochemistry, University of Bristol, Bristol, U.K.

Both positive and negative regulatory interactions between the protein kinase C (PKC) and insulin signaling pathways have been demonstrated previously by a number of investigators. In this study these regulatory interactions between the PKC and insulin signaling pathways have been further examined. Stable transfectants which overexpress the insulin receptor (IR) and either PKCα, PKCβI, PKCγ, or PKCε have been isolated and compared with parental CHO.T cells overexpressing only the IR. The parental and PKC transfected cell lines all express approximately 10^6 insulin receptors, indicating that overexpression of PKC did not affect IR levels. In their respective transfectants, tritiated phorbol ester binding showed that PKCα and PKCγ expressed 5 fold higher levels of PKC than parental cells while PKCβI and PKCε expressed 12 fold higher levels. In vivo ^{32}P-labeling experiments have been performed to compare the degree of insulin- and TPA-stimulated phosphorylation of the IR between the parental and PKC transfected cells. When all cells were treated with 1 μM insulin, the levels of ^{32}P incorporation into the IR were similar. Also there was no difference in the levels of phosphoserine, phosphothreonine or phosphotyrosine between the IR of the parental and PKC transfected cells. Therefore, insulin does not increase PKC activity in the transfected cells.

However 1 μM TPA treatment resulted in a 3 fold increase in ^{32}P incorporation into the IR and a 3-5 fold increase in serine and threonine phosphorylation in cells overexpressing PKCβI and PKCγ but not in the parental cells. There was no increase in IR ^{32}P incorporation or in serine or threonine phosphorylation in the PKCα and PKCε transfectants. These results further support the hypothesis that PKC can directly regulate IR serine and threonine phosphorylation and indicate that this effect is specific to PKCβI and PKCγ.

H 214 SIGNAL TRANSDUCTION PATHWAY OF IFNα2 INVOLVES TYROSINE PHOSPHORYLATION OF THE α SUBUNIT OF ITS REEPTOR, Colamonici O.R., and Platanias L.C., Department of Medicine, Univ. of Chicago, 5841 S. Mariland Ave. Box 420, Chicago, IL 60637-1470.

The IFNα2-induced phosphorylation of the IFNα receptor (IFNαR) was studied in theIFNα-sensitive Daudi,H-929 and U-266 cell lines, and in the IFNα-resistant U-937 cell line. In all IFNα-sensitive cell lines we detected a specific 110 kDa band after IFNα2 treatment, that corresponds to the phosphorylated α subunit of the IFNαR. Phosphorylation of the receptor on tyrosine was observed as early as 1 min after addition of IFNα2, reached its peak at 10 min, and gradually decreased being undetectable at 30 min. Phosphorylation could be induced with as little as 10 U/ml of IFNα2, and reached its plateau at 1,000 U/ml. Preincubation of the cells with Genistein (100 μg/ml), a tyrosine kinase inhibitor, blocked the tyrosine phosphorylation of the receptor. No effect on the intensity or timing of the IFNαR phosphorylation was observed when phosphatase blockers were used.Interestingly, the phosphorylation occurs even at 4ºC. At this temperature, the IFNαR showed hyperphosphorylation with decreased electrophoretic mobility that remained unchanged for up to 45 min. In the IFNα-resistant U-937 cell line no tyrosine phosphorylation could be detected upon IFNα2 binding. Study of the down modulation of the receptor by flow cytometry using the anti-IFNαR antibody IFNaR3, showed that U-937 cells failed to down regulate the IFNαR after IFNα2 treatment. Our data suggest that: 1) an early event in the signal transduction pathway of IFNα is the phosphorylation of its receptor on tyrosine; 2) in U-937 the resistance to the antiproliferative and antiviral effects of IFNα is associated with lack of phosphorylation and lack of down modulation of the IFNαR.

H 215 THE *Met*/HGF-SF RECEPTOR. Paolo M. Comoglio, Riccardo Ferracini, Lucia Gandino, Luigi Naldini and Carola Ponzetto, Department of Biomedical Sciences & Oncology, University of Torino, School of Medicine, 10126 Torino, Italy

Hepatocyte Growth Factor (HGF) and Scatter Factor (SF) are indistinguishable molecules produced by the stromal fibroblats and non-parenchimal cells of many organs and also present in serum. HGF-SF exerts an array of activities on epitelial cells, i.e. mitogenesis, dissociation of epithelial sheets, stimulation of cell motility, and promotion of matrix invasion. The $p190^{MET}$ heterodimer is the high affinity receptor for HGF-SF ($K_d = 0.25$ nM) on the surface of its target cells. This is proved by: (1) copurification of high affinity ligand binding activity with $p190^{MET}$, (2) chemical cross-linking of the radiolabelled ligand to the $p190^{MET}$ β subunit (3) reconstitution of a high-affinity binding site for HGF-SF into insect cells infected with a recombinant baculovirus carrying the *MET* cDNA.

HGF-SF binding triggers tyrosine autophosphorylation of the $p190^{MET}$ β subunit both in intact cultured cells and *in vitro* with the purified protein. Autophosphorylation upregulates the kinase activity of $p190^{MET}$, increasing the V_{max} of the phosphotransfer reaction. The major phosphorylation site has been mapped to Tyr 1235. Transmodulation of the HGF-SF receptor can occur through distinguishable pathways involving protein kinase C activation or increase in the intracellular Ca^{2+} concentration. Both lead to the phosphorylation of serine residue(s) in a unique tryptic phosphopeptide of $p190^{MET}$ and to a decrease in its tyrosine phosphorylation and kinase activity. HGF-SF acts as a growth or motility factor through the single *MET* receptor in different target cells.

H 216 Identification of Novel Protein Tyrosine Phosphatases in Mouse Mammary Tissue by PCR Amplification. **David L. Dankort and William J. Muller. The Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton, ON, Canada L8S 4K1**

Studies of acute transforming viruses lead to the notion that tyrosyl phosphorylation plays a pivotal role in tumorigenesis. These events are regulated by both tyrosine kinases (PTKs) and phosphatases (PTPs). We have utilized the polymerase chain reaction (PCR) using degenerate oligonucleotide primers constructed from conserved regions within the known PTPs to amplify and clone novel PTPs from normal and malignant mouse mammary tissue. A preliminary screen of the partial cDNAs revealed that we had isolated the mouse homolog to LAR as well as six potentially novel PTPs. To assess expression pattern of these clones, RNAse protection analyses were carried out using organ derived RNA. Preliminary expression data indicates that three (PTP M1, M2, and M3) of the four clones analyzed were expressed in the mammary gland while the fourth (PTP Br1), cloned from a mammary tumor, was expressed in the adult brain as well as the developing embryo. In addition to the mammary gland, other sites of expression include; thymus and spleen (PTPM1), brain, kidney, lung and heart (PTPM2), and a ubiquitously expressed PTPM3 clone. The functional and structural characteristics of these novel PTPs and their role in mammary gland development and tumorigenesis will be presented.

H 217 TYROSINE KINASE INDUCED INCREASES IN PHOSPHATIDYLINOSITOL KINASE ACTIVITY AND DNA SYNTHESIS IN IGF-I AND LUTEINIZING HORMONE (LH) TREATED BOVINE LUTEAL CELLS, John S. Davis and Aruna Chakravorty, The Women's Research Institute, Dept of Ob/Gyn, Univ of Kansas School Medicine-Wichita, and VA Medical Center, Wichita, KS 67214.

Polypeptide growth factors like insulin-like growth factor (IGF-I) modulate diverse cellular metabolic pathways and stimulate cell growth and differentiation. IGF-I-specific cell surface receptors possess intrinsic protein tyrosine kinase (pTK) activity. IGF-I-induced activation of receptor pTK initiates a program of intracellular events leading to the phosphorylation of several substrates including the receptor itself. The present study investigates phosphorylation of the IGF-I receptor and the mitogenic response in bovine luteal cells. Luteal cells were routinely cultured for 18 hr in the absence or presence of IGF-I (100 ng/ml) and [^{3}H]thymidine (TdR) incorporation into cellular DNA was measured during the last 4 hr. IGF-I, alone, increased TdR incorporation in a concentration dependent manner, reaching a maximum between 18-24 hr. LH had no effect alone. TdR incorporation was increased synergistically (10-20 fold) by concomitant treatment with LH (0.3 ng/ml) and IGF-I. Genistein (20 μM), a reversible inhibitor of pTK, significantly inhibited the increase in TdR incorporation. IGF-I induced autophosphorylation of WGA-purified IGF-I receptors was shown by SDS-PAGE and autoradiography. The pTK activity of the WGA-purified receptor was also demonstrated by the ability to increase phosphorylation (3-4 fold) of the substrate (poly)glu:tyr(4:1) following treatment with IGF-I. Phosphorylation of other substrates involved in signaling pathways was shown by enhanced phosphatidylinositol (PI) kinase activity after immunoprecipitation with an anti-phosphotyrosine antibody (PY20). Treatment with IGF-I for 30 min increased the amount of PI-kinase by 2-3 fold. LH alone had no effect on PI kinase but when incubated together with IGF-I a synergistic increase (3-4 fold) was observed. These results suggest that pTK activity is essential for DNA synthesis in bovine luteal cells, possibly by receptor autophosphorylation and phosphorylation of several protein substrates regulating cellular PI metabolism. Supported by NIH, VA, WRI, Wesley Foundation.

H 218 EGF-INDUCED AMINO-TERMINAL CLEAVAGE OF THE EGF RECEPTOR OCCURS BETWEEN EXTRACELLULAR DOMAINS 2 AND 3, Stuart Decker and Tania Habib. Signal Transduction, Parke-Davis, 2800 Plymouth Rd., and the Department of Microbiology and Immunology, University of Michigan, Ann Arbor, MI 48106.

We have previously reported that EGF causes an internalization-dependent proteolytic processing of the EGF receptor resulting in the generation of a Mr=120,000 receptor form truncated at the amino terminus. Sequencing of the amino terminus of the Mr=120,000 form from EGF treated A431 cells revealed two closely nested cleavage sites which lie in a region rich in basic residues at the boundary of extracellular domains 2 and 3; one site at residue 306 and one at residue 309. Through site directed mutagenesis, a receptor construct truncated at residue 308 was made and expressed in NIH-3T3 cells. Cells expressing this receptor form were morphologically transformed in an EGF independent manner, but did not grow in soft agar. These cells exhibited no binding of EGF. The Mr=120,000 truncate was constitutively autophosphorylated on tyrosine residues and possessed tyrosine kinase activity in vitro. These data suggest a potential role for this truncated receptor form in transmembrane signalling by the EGF receptor.

H 219 PROTEIN TYROSINE PHOSPHORYLATION AND THE NEUTROPHIL RESPIRATORY BURST, Donald L. Durden*, Henry Rosen, Bryce R. Michel and Jonathan A. Cooper, *Fred Hutchinson Cancer Research Center, 1124 Columbia St., Seattle, WA 98104.

Neutrophils stimulated with PMA, insoluble immune complexes (IC) or an opsonized oil emulsion (OP) undergo a dramatic increase in oxygen consumption termed the respiratory burst. In this report we examined the role of protein tyrosine phosphorylation in the regulation of the neutrophil respiratory burst. Antiphosphotyrosine immunoblotting of detergent extracts from IC or OP stimulated PMNs shows that Fc-receptor stimulation is associated with the tyrosine phosphorylation of specific cellular proteins whose apparent molecular weights are 105, 84, 76, 58, 52, 48, 45, 42, 34, 29 and 17 kilodaltons. In contrast to IC and OP stimulation, PMA stimulation of neutrophils was not associated with increased tyrosine phosphorylation of cellular proteins. Pretreatment of neutrophils with vanadate (2 mM), a protein tyrosine phosphatase inhibitor, inhibited the respiratory burst of neutrophils stimulated with PMA, IC and OP (70, 75 and 90% inhibition, respectively), but had no effect on Fc-receptor mediated phagocytosis, indicating that the inhibition of the respiratory burst was not due to general toxicity. Vanadate treatment induced the tyrosine phosphorylation of an 82 kd protein in resting cells and resulted in an increase in the phosphotyrosine response to PMA, IC and OP. An examination of the kinetics of the vanadate effect shows that the phosphorylation of an 82 kd protein correlated negatively with the neutrophil respiratory burst. The data suggest that protein tyrosine phosphorylation may play a role in the regulation of the neutrophil respiratory burst and that the phosphorylation state of these proteins in neutrophils is regulated by the activity of protein tyrosine phosphatases. The tyrosine phosphorylation of p82 may be involved in the regulation of the neutrophil respiratory burst.

H 220 SIGNALLING VIA TYROSINE PHOSPHORYLATION IN HEMOPOIETIC CELLS. Vincent Duronio and John W.Schrader. The Biomedical Research Centre, 2222 Health Sciences Mall, U.B.C., Vancouver, B.C., V6T 1Z3, Canada.

Receptors for numerous hemopoietic growth factors have been shown to be members of a new receptor superfamily. A characteristic feature of these receptors is that in most cases, stimulation of tyrosine phosphorylation in response to the cytokines has been described, but the tyrosine kinases have not been positively identified. We have examined the tyrosine phosphorylation events that occur in response to a number of these factors, with particular emphasis on interleukin-3 (IL-3) and granulocyte-macrophage colony stimulating factor (GM-CSF). Both IL-3 and GM-CSF stimulate a similar pattern of polypeptides. The phosphorylated substrates are being purified by immunoaffinity chromatography in order to characterize them. A major substrate in both cases has an apparent molecular weight of approx. 135,000 at early times (1-2 min) following stimulation and approx. 150,000 at later times (5-10 min). In the case of IL-3 stimulation, this substrate was shown to be the low affinity IL-3 receptor, or the AIC-2A polypeptide, which normally has an Mr = 125,000. The related protein phosphorylated in response to GM-CSF is probably the AIC-2B polypeptide.

Analysis of other potential tyrosine kinase substrates has revealed that one of the subtrates is a member of the MAP kinase family of serine/threonine kinases. Antibodies to PLC-gamma and GAP have been used to show that these enzymes are not phosphorylated on tyrosine in response to the growth factors. The negative result with GAP was particularly interesting since we have found that at least five different hemopoietic growth factors can activate p21ras. Kinase inhibitors were used to show that the activation of p21ras required tyrosine kinase activity, but not protein kinase C.

H 221 THE C.ELEGANS GENE, DAF-4, IS A RECEPTOR SERINE KINASE SIMILAR TO THE ACTIVIN RECPTOR. Miguel Estevez, Patrice S. Albert, and Donald L. Riddle. Molecular Biology Program, University of Missouri, Columbia, MO, 65211.

Starvation and overcrowding of *C. elegans* during the first larval stage induces formation of developmentally arrested dauer larvae at the second molt. When conditions favorable for growth are found they resume development to adulthood. Mutants affected in the decision to form dauer larvae are either dauer-constitutive, which form dauer larvae in abundant food, or dauer-defective, which cannot form dauer larvae when starved. Interactions between specific dauer-constitutive and dauer-defective mutants have been used to construct a pathway for gene action. *daf-4*, a dauer-constitutive gene near the end of the pathway has recently been cloned by transposon tagging. Sequences flanking the transposon insertion site were used to isolate genomic and cDNA clones. Microinjection of one of the genomic clones into the germline of *daf-4* mutants transforms their progeny to wild-type, demonstrating that the cloned DNA conveys *daf-4* function. The cDNA's encode a kinase 33% identical to the kinase domain of the *daf-1* transmembrane receptor kinase (Georgi et al., Cell 61: 635, 1990), and 40% identical to the kinase domain of the activin receptor (Matthews and Vale, Cell 65: 973, 1991). A *daf-4* hydrophobicity plot suggests it has transmembrane and extracellular domains. It is intriguing that the putative extracellular domains of the *daf-4* and *daf-1* proteins share a short sequence motif with the activin receptor and the TGF-β1 binding protein. It is possible that the *daf-1* and *daf-4* proteins are *C. elegans* homologs of the mammalian type I and type II TGF-β receptors previously proposed by Laiho et al. (JBC 265: 18518, 1990). Similarities in the non-coding regions of *daf-1* and *daf-4* suggest that they could be coordinately regulated.

H 222 STRUCTURE AND FUNCTION OF CHICKEN PROTEIN TYROSINE PHOSPHATASES, Siyun Fang, Hisataka Sabe, Christopher Marshall and Hidesaburo Hanafusa, Laboratory of Molecular Oncology, The Rockefeller University, New York, NY 10021

Protein tyrosine kinases are known to be involved in oncogenic processes induced by certain oncogenes. Protein tyrosine phosphatases (PTPases) may also play important roles in regulating these processes. We recently cloned and sequenced two chicken PTPases: ch-PTPα and ch-PTPx. Both are receptor-type PTPases. ch-PTPα is a homolog of mammalian PTPα, sharing 94% identity in their amino acid sequences within transmembrane spans and intracellular domains, while ch-PTPx is 67.5% homologous to hLCA in these two regions. ch-PTPα is expressed ubiquitously, but more highly in chicken brain, while ch-PTPx is primarily detected in chicken hematopoietic cells. Both proteins showed tyrosine-specific PTPase activity when they were expressed in bacteria.

It is proposed that PTPases can participate in regulating cell growth and differentiation, either negatively or positively. For example, dephosphorylation of cellular proteins by PTPase in v-Src-induced transformed cells may suppress transformation or cell growth. On the other hand, PTPase may dephosphorylate Tyr527 of c-Src to activate its protein tyrosine kinase, thus promoting cell growth. To explore these possibilities, we have introduced the cytoplasmic domain of ch-PTPx into cells overexpressing c-Src, v-Src or v-Crk. The results will be discussed.

H 223 A DROSOPHILA TYROSINE KINASE SIMILAR TO THE MAMMALIAN CSF-1 RECEPTOR, Anthony W. Ferrrante, Jr. and E. Richard Stanley, Department of Developmental Biology & Cancer, Albert Einstein College of Medicine, Resnick Campus, Bronx, NY 10461

Over the last ten years it has become apparent that receptor tyrosine kinases (RTK) play critical roles in many developmental processes. To study the function of RTK most researchers have relied on tissue culture and biochemical techniques and have not exploited genetic approaches. Indeed, most reported tyrosine kinases are mammalian or avian proteins, and as such do not lend themselves to rapid analysis by genetics. This lead us to search in genetically more pliable organisms for a model RTK to study.

Using a simple amplification strategy we have identified in *Drosophila melanogaster* several tyrosine kinase receptors, in particular, receptors which most broadly belong to the CSF-1 Receptor family. Among these RTK's were such previously characterized receptors as *torso* and an FGF receptor homolgue. In addition, several novel receptors, including a second FGF receptor and a *ret* homologue have been identified using this strategy. One amplification clone, TK17, shows greatest similarity to the mammalian CSF-1 Receptor . Flies express TK17 mRNA during late larval development and pupation, possibly involving it in the development of an imaginal disc. By *in situ* hybridization to polytene chromosomes, TK17 appears to map to 53A3, a region to which no other RTK is known to map. Cloning of a full length cDNA and a search for mutations are underway.

H 224 ENZYME ACTIVITIES ASSOCIATED WITH THE PLAZMA MEMBRANE SUBSTRATE OF THE INSULIN RECEPTOR, H.J. Goren, D. Boland and Q. Fei, Department of Medical Biochemistry, University of Calgary, Calgary, Canada T2N 4N1.

Wheat germ agglutinin (WGA)-purified insulin receptor preparations phosphorylate themselves and a 180 kDa protein (p180). To determine whether the plasma membrane insulin receptor substrate, p180, functions in insulin signalling, p180 biological properties were studied. WGA-purified rat liver membranes were phosphorylated [$MnCl_2$ 5mM, 50μM ATP] in the absence or presence of 10^{-8}M insulin. Antiphosphotyrosine-immunoprecipitated proteins were incubated with phosphatidyl-inositol (PI) and ATP. Thin layer chromatography demonstrated that some PI-3'-phosphate was formed with insulin-stimulated (but not with basal) tyrosine-phosphorylated protein pellets. Resuspended antiphosphotyrosine-immunoprecipitated proteins were incubated 30 min, 4°C with insulin-stimulated autophosphorylated WGA-purified rat liver membranes. Proteins were then immunoprecipitated with antiphosphotyrosine and analyzed in dodecyl sulphate gel electrophoresis. The addition of tyrosine-phosphorylated p180 to the phosphorylation reaction decreased phosphorylated insulin receptor β-subunit. Sepharose 4B chromatography permitted separation of insulin receptors and p180. Addition of ATP and $MnCl_2$ to p180 did not yield phosphorylated protein nor was poly(Glu_4,Tyr) phosphorylated with its addition. These findings suggest: 1) that p180 is not a tyrosine kinase, 2) that p180 has tyrosine phosphatase activity, and 3) that some PI 3'-kinase activity is associated with tyrosine-phosphorylated p180 or insulin receptor. Accordingly, p180 function in insulin signalling could include: regulation of insulin receptor tyrosine kinase activity by dephosphorylating it, or recruitment of cytoplasmic enzymes to the plasma membrane for the purpose of generating second messengers. (Supported by MRC Canada).

H 225 BIOCHEMICAL COMPARISONS OF THE NORMAL AND ONCOGENIC FORMS OF THE INSECT CELL-EXPRESSED NEU TYROSINE KINASE, Pamela M. Guy, Kermit L. Carraway, III, and Richard A. Cerione, Department of Biochemistry, Cell and Molecular Biology, Cornell University, Ithaca, NY 14853.

The tyrosine kinase activity of the neu protein may be activated by a point mutation within its transmembrane region. Using three different forms of neu expressed in insect cells via baculovirus infection, we have examined the role of the transmembrane region in the regulation of the neu protein tyrosine kinase activity. One form consisted of the complete tyrosine kinase domain but lacked the extracellular and transmembrane regions. The other two forms consisted of the tyrosine kinase domain, the transmembrane region, and forty amino acids of the extracellular domain. One of these amino-terminally truncated forms of neu contained the normal valine residue at position 664 within the transmembrane region, while the other contained the oncogenically activating glutamic acid residue at this position. We have found that the presence of the transmembrane region of neu suppresses the tyrosine kinase activity of this receptor. In addition, we have found that the oncogenically activating point mutation within the transmembrane region stimulates the tyrosine kinase activity of the neu protein by allowing it to more effectively utilize physiological divalent metal ions in its catalytic mechanism.

H 226 STEEL FACTOR (C-KIT LIGAND) AND GM-CSF USE BOTH UNIQUE AND COMMON SIGNAL TRANSDUCTION PATHWAYS. Michael Hallek, Brian Druker, Eva M. Lepisto, Timothy J. Ernst, Keiko Okuda & James D.Griffin. Dana-Farber Cancer Institute, Boston, MA 02115, USA.

Steel factor (SF) supports the growth and differentiation of human hematopoietic progenitor cells in synergy with a variety of hemopoietins including GM-CSF. In an effort to characterize the mechanism of interaction of these factors, we have investigated their effects on the human factor-dependent cell line MO7. Proliferation of this cell line is induced by either GM-CSF or SF alone, but the maximum rate of proliferation is increased synergistically (3-6 fold) with combinations of SF and GM-CSF. This synergy was not due to an SF-induced increase in GM-CSF receptor number or affinity. The effects of SF and GM-CSF on the activation of several intracellular signal transduction pathways were assessed. Each of the cytokines stimulated rapid tyrosine phosphorylation of a number of substrates. GM-CSF induced tyrosine phosphorylation of several phosphoproteins with mol. weights of 150, 125, 93, 63, 55, 44, and 42 kDa; proteins phosphorylated in response to SF had mol. weights of 140-150, 116, 94, 63, 44 and 42 kDa. A potential overlap between GM-CSF and SF was observed for 42, 44, and 63-kDa proteins. Tyrosine phosphorylation induced by both factors was rapid, transient (0.5 to 15 min) and concentration-dependent (peak at 50-100 ng/ml for SF; 10-100 ng/ml for GM-CSF). Some of the substrates phosphorylated in response to these growth factors were identified by immunoblotting with monospecific antibodies (table 1). Additionally, the induction of the transcriptional factors c-jun, c-myc and c-myb by either factor was assessed by Northern blotting (table 2).

Table 1. Protein tyrosine phosphorylation induced by GM-CSF or SF

Signal transduction intermediate	mol.w. (kDa)	GM-CSF	SF
c-kit	145	-	+
phospholipase C-γ (PLC-γ)	145	-	+
mitogen associated protein kinase (MAPK)	42, 44	+	+
Raf-1 (serine phosphorylation only)	70-75	+	+

Table 2. Induction of mRNA by GM-CSF or SF (at 30 and 90 min)

	GM-CSF	SF
c-myc	+	-
c-myb	+	-
c-jun	-	-

While SF had no effect on c-myc and c-myb at 30 and 90 min in contrast to GM-CSF, a slight increase of c-myc and c-myb mRNA expression was observed after 22 h suggesting that SF may have delayed effects on these proto-oncogenes. In conclusion, the synergy between SF and GM-CSF may result from the activation of common signal transduction intermediates like MAPK and Raf-1, as well as of unique pathways such as activation of c-kit, PLC-γ, c-myc, and c-myb.

H 227 THE MURINE RECEPTOR TYROSINE KINASE *nuk* IS CONCENTRATED IN A SUBSET OF CELL-CELL JUNCTIONS DURING EMBRYOGENESIS

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We describe the cloning and embryonic expression pattern of a new murine receptor tyrosine kinase called *nuk* (neural kinase). *nuk* is very similar in structure to the the growing subclass of receptor tyrosine kinases which include *eph*, *eck*, and *elk*. Common features of these proteins include a cysteine-rich subdomain and fibronectin type III repeats in the extracellular domain and a fairly well conserved intracellular catalytic domain.

To begin to understand the developmental function of this receptor tyrosine kinase, the expression of *nuk* mRNA and protein during embryogenesis has been investigated using whole-mount RNA *in situs* and whole-mount immunohistochemical protocols. The results indicate that *nuk* is highly expressed in the neuroectoderm as early as 8.5 days post coitum. Later, during formation of the brain and neural tube, *nuk* is highly expressed in the telocoel (primative forebrain), midbrain flexure, optic vesicle and in the roof plate of the neural tube. In addition, *nuk* is expressed at elevated levels in the base of the developing heart.

Immunohistochemical staining with anti-*nuk* antibodies revealed that endogenous *nuk* protein is asymetrically located at the cell surface. The staining pattern is very filamentous and appears to be localized to the extracellular matrix separating different cell/tissue types. Electron microscopy studies indicate *nuk* protein is concentrated at some, but not all, cell-cell junctions. The presence of fibronectin type III repeats and the unusual localization of *nuk* protein suggest that this receptor tyrosine kinase may function to control cell-cell interactions during murine embryogenesis.

H 228 EPIDERMAL GROWTH FACTOR RECEPTOR INCREASES PHOSPHOLIPASE C-γ1 ACTIVITY IN VITRO INDEPENDENTLY OF TYROSINE PHOSPHORYLATION, S.M.Teresa Hernández-Sotomayor and Graham Carpenter, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146

Phospholipase C-γ1 (PLC-γ1) is a substrate for the epidermal growth factor (EGF) receptor tyrosine kinase. In intact cells or in vitro, tyrosine phosphorylation increases the catalytic activity of PLC-γ1 suggesting that at least in part, catalytic activation of PLC-γ1 is regulated by tyrosine phosphorylation. Several reports also suggest that there is a physical association between PLC-γ1 and the EGF receptor, though this is based only on coprecipitation experiments. In an effort to investigate the possible functional role of receptor:PLC-γ1 complexes, we have measured PLC-γ1 activity in the presence or absence of the EGF receptor. Immunoprecipitates of PLC-γ1 from control A-431 cells were incubated with purified EGF receptor and ATP. After a 60 min incubation, the EGF receptor was removed and the activity of both phosphorylated and non-phosphorylated forms of PLC-γ1 was measured in the absence or presence of re-added EGF receptor without the presence of ATP. We found that the EGF receptor increases PLC activity two-fold in the absence of ATP. Both the non-phosphorylated and phosphorylated forms of the purified EGF receptor increased the activity of PLC-γ1. The EGF receptor stimulation is particularly evident with the non-phosphorylated form of PLC-γ1. Our results suggest that EGF-receptor association with PLC-γ1 may be the first step in the activation of this enzyme, at least in vitro.

Supported by PO1 CA 43720 and Fogarty Fellowship TW04600.

H 229 IDENTIFICATION OF A NOVEL 140 KD NERVE GROWTH FACTOR BINDING PROTEIN IN RAT PC12 CELLS. Cornelia Hertel, Michelle McCormack, Robert Schubenel, and Deborah S. Hartman. F. Hoffmann- La Roche AG, Pharma Division, Preclinical Research, Basel, Switzerland.

Rat pheochromocytoma (PC12) cells express two distinct nerve growth factor receptors (NGFRs), $p75^{NGFR}$ and $p140^{trk}$. When expressed individually in heterologous cell lines, both $p75^{NGFR}$ and $p140^{trk}$ bind ^{125}I-NGF with low affinity. However co-expression of $p75^{NGFR}$ and $p140^{trk}$ is reported to give rise to both high and low affinity NGF binding sites (*Nature* 350, 678-683, 1991). To determine whether one or both of these proteins participate in the formation of the high affinity NGFR, we have established a method to selectively crosslink ^{125}I-NGF bound to $p75^{NGFR}$ or $p140^{trk}$ in PC12 cells where they are co-expressed.

We find that BS^3 crosslinks ^{125}I-NGF to $p140^{trk}$, and to two protein bands with apparent molecular weights greater than 200 kD on SDS polyacrylamide gels. Both high molecular weight bands, named $p250^{trk}$ and $p300^{trk}$, are immunoprecipitated by a polyclonal antiserum (anti-trk) raised against the cytoplasmic domain of the trk oncogene. $p250^{trk}$ and $p300^{trk}$ are not immunoprecipitated, however, by mAb192, a monoclonal antibody specific for the $p75^{NGFR}$ protein. $p140^{trk}$, $p250^{trk}$ and $p300^{trk}$ in intact PC12 cells exhibit slow offrates, with no ligand dissociation detectable after 20 minutes in the presence of 400-fold excess NGF.

BS^3 does not crosslink ^{125}I-NGF to the $p75^{NGFR}$ protein. However, $p75^{NGFR}$ protein can be crosslinked to ^{125}I-NGF using EDC. $p75^{NGFR}$ can be immunoprecipitated by mAb192, and exhibits a very fast ligand dissociation rate in the presence of cold NGF. EDC also crosslinks NGF to a novel 140 kD protein, named $p140^{EDC}$, which is not immunoprecipitated by trk antiserum or by mAb192. $p140^{EDC}$ exhibits a very fast ligand dissociation rate, which further distinguishes it from $p140^{trk}$.

In summary, by using crosslinkers and antibodies which specifically recognize $p75^{NGFR}$ or $p140^{trk}$, we have identified a novel NGF binding protein, $p140^{EDC}$, which may also play a role in formation of the high affinity NGFR. The ability of chemical crosslinkers to distinguish between $p75^{NGFR}$ and $p140^{trk}$ ligand-receptor complexes suggests divergent structures of the NGF binding domains in these two molecules.

H 230 IL-2 Dependent Proliferation Of LAK Cells Is Accompanied By Protein Tyrosine Phophorylation Events. Ivan D. Horak[1], Angus J. Grant[2], Eva M. Horak[1], Erich Roessler[2], Thomas A. Waldmann[2] and Joseph B. Bolen[3]. Clinical Pharmacology Branch[1], Metabolism Branch[2], National Cancer Institute, Bethesda, MD 20892; Department of Molecular Biology[3], Bristol-Myers Squibb Pharmaceutical Research Institute, NJ 08543

IL-2 plays a central role in the growth of T-cells, the generation of lymphokine activated killer (LAK) cells from large granular lymphocytes (LGLs), and also affects the activity of monocytes. The interaction of IL-2 with its intermediate affinity (IL-2Rβ) chain also is sufficient to activate LGLs and monocytes. However, in order for IL-2 to deliver a proliferative signal to T-cells and LGLs, but not to monocytes, both the IL-2Rα (low affinity) and IL-2Rβ (intermediate affinity) chains must be present to form the high affinity receptor complex. Recent studies of several T-cell lines have demonstrated that IL-2 provokes a rapid increase in tyrosine phosphorylation of the IL-2Rβ chain and several other proteins (42, 56, 57, 63, 72, and 92kDa) even though neither the IL-2Rα chain nor the IL-2Rβ chain possess intrinsic protein tyrosine kinase activity. We have recently found that the T lymphocyte specific protein tyrosine kinase $p56^{lck}$ is involved in IL-2-induced tyrosine protein phosphorylation events. In order to elaborate on the role of $p56^{lck}$ in β chain transduced proliferative signals we analyzed human LGLs and monocytes which constitutively express high levels of IL- 2Rβ, but not IL-2Rα. The normal expression of $p56^{lck}$ appears to be limited to T-cells and LGLs but not to monocytes. We have found that while IL-2 increased the cytotoxic activity of monocytes and LGLs, was not accompanied by tyrosine phosphorylation events. Addition of IL-2 to LAK cells but not to LGLs resulted in diminished SDS/polyacrylamide gel mobility of a proportion of $p56^{lck}$. Activation of $p56^{lck}$ occurs only during IL-2-induced proliferative signals in activated LGLs (LAK cells) expressing both IL-2Rα and IL-2Rβ chains.

H 231 CALCIUM-DEPENDENT INCREASE IN TYROSINE KINASE ACTIVITY STIMULATED BY ANGIOTENSIN II. William R. Huckle and H. Shelton Earp, Lineberger Comprehensive Cancer Center, School of Medicine, University of North Carolina, Chapel Hill, NC 27599

Tyrosine phosphorylation of proteins is a regulatory mechanism usually associated with growth factors (e.g., EGF and PDGF) that act through tyrosine kinase (TK)-containing receptors or with several families of oncogenic, non-receptor TKs (e.g., *src* and *abl*). We have shown recently (Mol. Cell. Biol. 10:6290 [1990]) that treatment of WB liver epithelial cells with angiotensin II (AngII), vasopressin or epinephrine increases tyrosine phosphorylation by a Ca^{2+}-dependent mechanism. To test the involvement of TK activation in this novel response, we have examined TK activity in extracts of cells treated with Ca^{2+}-mobilizing agents. Since many known TKs autophosphorylate or associate with their phosphorylated substrates as a consequence of activation, we reasoned that activation of TKs by Ca^{2+}-mobilizing agents would be reflected by an increase in P-Tyr-associated TK activity. TK activity in anti-P-Tyr immunoprecipitates from control, EGF- or AngII-treated cells was assayed by incubation with 5 μM [γ-^{32}P]ATP, 30 mM Mg^{2+}, 1 mM Mn^{2+} and the synthetic TK substrate poly(Glu_4Tyr). *In WB cells, AngII treatment increased* TK activity to 150-200% of untreated levels. In the WB-derived cell line GN4, which exhibits a greater AngII-stimulated increase in endogenous tyrosine phosphorylation in intact cells (measured by immunoblotting), immune complex TK activity was increased to *400% of controls; EGF treatment of* either WB or GN4 cells produced comparable (4-fold) increases in TK activity. Similar AngII-stimulated enhancements of immune complex TK activity were observed when enolase was substituted for polyGluTyr as a substrate, or when no exogenous substrate was added. *In the latter case,* endogenous proteins of M_r 75-78 and 115-125 kDa were phosphorylated, producing a pattern very similar to that seen upon P-Tyr immunoblotting of cell lysates after AngII treatment. For both WB and GN4 cells, the AngII-stimulated increase in immune complex TK activity was transient (maximal at 45-60 sec) and closely paralleled the transient increase in endogenous protein P-Tyr detected by immunoblotting of whole lysates. Pre-loading WB or GN4 cells with the Ca^{2+} chelator BAPTA, which prevents AngII-stimulated Ca^{2+} mobilization (measured by Fura-2 fluorescence), totally abolished AngII-stimulated immune complex TK activity, but did not inhibit EGF-stimulated activity. These correlations between stimulated cellular P-Tyr levels and immune complex TK activity suggest that activation of one or more TKs is an important element in Ca^{2+}-dependent stimulation of tyrosine phosphorylation.

H 232 CHARACTERIZATION OF MCF-7 BREAST CARCINOMA CELLS SINGLE OR DOUBLE TRANSFECTED WITH *c-erb B2* OR EGFR EXPRESSION VECTORS. Francis G. Kern, Ellie Y. Liu, David Miller, and Denise Chen. Lombardi Cancer Center, Georgetown University Medical Center, Washington DC 20007. Both EGFR and ERB B2 have structures common to transmembrane receptor tyrosine kinase molecules. Overexpression of either molecule can be tranforming in rodent fibroblast systems where "crosstalk" involving both receptors has also been demonstrated . Overexpression of either *c-erb B2* or EGFR is associated with a poorer prognosis in human breast cancer. EGFR overexpression is rarely found and *c-erb B2* overexpression is less frequently found in tumors that also express estrogen receptor raising the possibility that overexpression may be associated with the progression of breast cancer from a hormone dependent to a hormone independent state. To test this hypothesis we have derived transfectants of the estrogen responsive and antiestrogen senstive MCF-7 cell line that express elevated levels of either ERB-B2 or EGFR protein or co-overexpress elevated levels of both proteins. Western blotting with antiphosphotyrosine antibodies indicates expression of appropriately sized functional molecules. Overexpression is unstable when cells are maintained in media containing either fetal calf serum or charcoal stripped calf serum (CCS) supplemented with 17-beta estradiol (E2). However, when cells are maintained in CCS media devoid of E2, there is a selection for cells that overexpress either receptor. Both phenomenon involve signal transduction by the epidermal growth factor receptor since these effects are not observed when the cells are transfected with an expression vector containing a point mutation that inactivates the kinase. Under E2 deprived conditions all of the transfectants are stimulated by the addition of 1.0 ng/ml of EGF including control clones of MCF-7 cells transfected with the expression vector lacking any cDNA insert. While higher doses of EGF remain stimulatory to the control and ERB B2 transfected cells, they are inhibitory to the cells overexpressing EGFR or both EGFR and ERB B2 with the dose resonse shifted to the right in the double transfectants.

H 233 THE *TRK* FAMILY OF NEUROTROPHIN RECEPTORS Rüdiger Klein, Fabienne Lamballe, Shuqian Jing, Venkata Nanduri, Peter Tapley and Mariano Barbacid, Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543.

The NGF family of neurotrophic factors (NGF, BDNF, NT-3 and NT-4) recognizes at least two distinct classes of cell surface receptors. One of these receptors is a cysteine-rich glycoprotein, $p75^{LNGFR}$, which contains a short cytoplasmic domain devoid of informative structural motifs. $p75^{LNGFR}$ binds each of the above neurotrophins with the same low affinity ($K_d \sim 10^{-9}$ M) and does not mediate most of their biological responses. Recent studies have indicated that the NGF family of neurotrophins also binds to the *trk* family of tyrosine protein kinase receptors. The *trk* gene was first identified as the normal allele of an oncogene present in a human colon carcinoma biopsy. The related *trk*B and *trk*C genes were subsequently isolated by screening brain cDNA libraries at low stringency. Unlike $p75^{LNGFR}$, these *trk* receptors show binding specificity. Whereas NGF only binds to the product of the *trk* proto-oncogene, $gp140^{trk}$, BDNF specifically recognizes $gp145^{trk}$B, the tyrosine protein kinase receptor encoded by the *trk*B locus. The *trk*C gene product, $gp145^{trk}$C, appears to be the primary receptor for NT-3, a neurotrophin that can also interact with $gp140^{trk}$ and $gp145^{trk}$B. Recent studies regarding the binding specifity of the recently identified NT-4 protein will be presented.

The interaction between these neurotrophins and their cognate *trk* receptors elicits a variety of biological responses including (i) phosphorylation of the receptor in tyrosine residues; (ii) induction of c-Fos expression; (iii) induction of DNA synthesis (iv) survival and neurite outgrowth in PC12 cells and (v) morphologic transformation of NIH3T3 cells. None of these biological responses requires the presence of $p75^{LNGFR}$. Moreover, they are not affected by co-expression of this low affinity receptor. Finally, each of the *trk* tyrosine kinases binds their cognate neurotrophin(s) with high affinity ($K_d \sim 10^{-11}$ M), either in the presence or absence of $p75^{LNGFR}$. These findings strongly suggest that the *trk* family of tyrosine protein kinases are the high affinity receptors that mediate the known neurotrophic properties of the NGF family of neurotrophins.

H 234 The C-termial SH2 Domain of p85 Accounts for High Affinity and Specificity of the Binding of PI3-kinase to Phosphorylated PDGF β-receptor. Anke Klippel[1, 2], Jaime A. Escobedo[1, 2], Wendy J. Fantl[1] and Lewis T. Williams[1, 2], *Howard Hughes Medical Institute*[1], *Cardiovascular Research Institute*[2], *University of California San Francisco, San Francisco, CA 94143*

Upon stimulation by its ligand the platelet-derived growth factor (PDGF) receptor associates with the 85 kDa subunit of phosphatidylinositol 3-kinase (PI3-kinase). The 85 kDa (p85) protein contains two Src homology (SH) 2 domains and one SH3 domain. To define the part of p85 that interacts with the PDGF receptor, a series of truncated p85 mutants was analyzed for association with immobilized PDGF receptor *in vitro*. We found that a fragment of p85 that contains a single Src homology domain, the C-terminal SH2 domain (SH2-C), was sufficient for directing the high affinity interaction with the receptor. Half maximal binding of SH2-C to the receptor was observed at a SH2-C concentration of 0.1 nM. SH2-C, like full-length p85, was able to distinguish between wild-type PDGF receptor and a mutant receptor lacking the PI3-kinase binding site. An excess of SH2-C blocked binding of full-length p85 and PI3-kinase to the receptor, but did not interfere with the binding of two other SH2-containing proteins, phospholipase C-γ and GTPase activating protein. These results demonstrate that a region of p85 containing a single SH2 domain accounts both for the high affinity and specificity of binding of PI3-kinase to the PDGF receptor.

H 235 EARLY EVENTS IN THE CONTROL OF BREAST CANCER CELL GROWTH BY INSULIN-LIKE GROWTH FACTORS, David E. Lebwohl, Harry H. Hall and Neal Rosen, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

Insulin-like growth factors(IGF I and IGF II) are potent mitogens for breast cancer cells, and have been implicated in the pathogenesis of breast malignancy. IGFs are produced by the fibroblasts surrounding primary breast cancer cells. In later stages of tumor progression, tumors may grow independently of supporting cells which produce growth factors. Signal transduction by insulin and IGFs was investigated in various breast cancer cell lines. The three ligands stimulated the autophosphorylation of IGF-I receptors on tyrosine residues, as detected in antiphosphotyrosine immunoblots and by immunoprecipitation from ^{32}P-labeled cells. In addition, tyrosine phosphorylation of cellular substrates occurred within one minute of insulin/IGF stimulation, including several proteins with molecular weights of approximately 185kDa, similar to a recently cloned insulin receptor substrate IRS-1. Cell lines differ in both the size of their expressed IGF-I receptors, and in the substrates detected. These biochemical findings, as well as an analysis of other early events, will be correlated with the growth response of these cell lines to insulin and IGFs. Possible mechanisms of growth-factor independence will be discussed.

H 236 ACTIVITIES OF SPECIFIC CD45 ISOFORMS EXPRESSED IN TRANSGENIC MICE, Jamey D. Marth, Christopher J. Ong, Daniel Chui, Hung-Sia Teh and Pauline Johnson. The Biomedical Research Centre, and the Depts. of Medical Genetics, Biochemistry and Microbiology, 2222 Health Sciences Mall, University of British Columbia, Vancouver, B.C. Canada V6T 1Z3

Although CD45 is known to be a tyrosine phosphatase with receptor structure, the function and activity of this enzyme with respect to alternate extracellular exon usage and potential ligand-induced regulation remain undefined. To address the role of alternate CD45 isoforms in regulating immune system development and function, transgenic mice were created in which the B220 isoform of CD45, normally found at high levels only on the surface of B lymphocytes, was expressed in the thymocyte population using the thymus-specific *lck* proximal promoter. Intact cell surface B220 was observed in all transgenic thymocyte subsets. Epitope mapped isoform specific monoclonal antibodies were used to delineate CD45 isoform usage in control and transgenic thymocytes. By comparison to endogenous CD45 expression, transgenic B220 expression increased total CD45 levels by only 5-10%. The developmental profile of transgenic thymocytes revealed significant alterations in specific subpopulations in a manner suggesting that B220 accentuates T cell receptor signalling. Upon in vitro immunologic stimulation of specific thymocyte subsets by T cell receptor (CD3-antibody) crosslinking, B220+ thymocytes were hyperstimulatable, displaying a 10-fold increase in ^{3}H-thymidine uptake and a corresponding increase in interleukin-2 production over control cell values. This hyperstimulation appears dependent upon an increased LFA-1 driven cellular aggregation response. Experiments detailing immunoblotting and immunoprecipitation with antibodies to phosphotyrosine, tyrosine kinases and kinase substrates will be discussed, as will the molecular identities of proteins affected. Transgenic mice bearing CD45 constructs lacking the three extracellular exons contained in B220 (termed T200) are being analyzed presently. Taken together these data infer that, within the thymic milieu, B220 is an inappropriately regulated phosphatase and may functionally compete with cellular proteins, possibly specific CD45 isoforms, to effect the T cell receptor hyperstimulation observed. The above experiments and results will be discussed in the context of a proposed mechanism that regulates isoform specific CD45 function through the extracellular 'ligand binding/regulatory' domain.

H 237 ACTIVE SERINE/THREONINE KINASE RAF-1 IS ASSOCIATED WITH IL-2 RECEPTOR BETA CHAIN PRIOR TO IL-2 STIMULATION, Wlodzimierz Maslinski, Brian Remillard, Edward Hadro, Terry B. Strom, Department of Medicine, Harvard Medical School, Beth Israel Hospital and New England Deaconess Hospital, Boston, MA 02215

The interleukin-2 (IL-2) autocrine pathway is central for clonal proliferation of antigen stimulated T lymphocytes. Following stimulation with IL-2 rapid activation of protein tyrosine kinase, serine threonine kinase and phosphatidyl inositol 3-kinase (PI 3-kinase) ensue. The IL-2 receptor beta chain, which is responsible for initiating signal transduction, lacks a consensus sequence for a protein tyrosine kinase. Protein tyrosine kinase activity is due, at least in part, to recruitment of the cellular protein tyrosine kinase, $p56^{lck}$, to the IL-2 receptor following stimulation with IL-2. In addition, IL-2 induces rapid appearance in the cytosol of the activated, phosphorylated form of Raf-1, a serine/threonine kinase. Herein we report that prior to IL-2 stimulation enzymatically active Raf-1 is physically associated with the IL-2 receptor beta chain (p75) in T-cell blasts. Following stimulation with IL-2 enzymatically active Raf-1 dissociates from the IL-2 receptor complex into the cytosol. Genistein, a protein tyrosine kinase inhibitor, prevents the dissociation of Raf-1 from the ligand stimulated IL-2 receptor complex. These data favor a model of IL-2 receptor activation where an IL-2 activated protein kinase ($p56^{lck}$) associates with the IL-2 receptor complex and phosphorylates receptor bound Raf-1. Following tyrosine phosphorylation enzymatically active Raf-1 dissociates from the IL-2 receptor and migrates into the cytosol.

H 238 INTERLEUKIN-2 BINDING ACTIVATES A TYROSINE-PHOSPHORYLATED PHOSPHATIDYLINOSITOL-3-KINASE, Isabel Merida, Emilio Diez and Glen Gaulton, Department of Pathology and Laboratory Medicine, Division of Immunobiology, University of Pennsylvania, School of Medicine and Department of Cell Sciences, SmithKline Beecham Pharmaceuticals Philadelphia Pa 19104.

The biochemical signaling mechanisms of IL2-receptor were examined by studying IL2-dependent activation of phosphatidylinositol-3 kinase, and the relationship between IL2-receptor associated tyrosine kinase and phosphatidylinositol-3 kinase activities in the IL2-dependent murine T cell line CTLL-2. Phosphatidylinositol-3 kinase was isolated by immunoprecipitation with anti-phosphotyrosine antibodies, and kinase activity determined using an *in vitro* assay that measures the formation of phosphatidylinositol 3 phosphate. IL2 rapidly increased anti-phosphotyrosine immunoprecipitable phosphatidylinositol-3 kinase activity in the CTLL-2 line. Tyrosine phosphorylation of phosphatidylinositol-3 kinase was demonstrated by immunoabsorption with anti-phosphotyrosine antibody, and was coincident with activation of IL2-receptor associated tyrosine kinase. Incubation of cells with genistein, a selective tyrosine kinase inhibitor, blocked IL2-dependent activation of receptor associated tyrosine kinase and phosphatidylinositol-3 kinase activities. Finally, the addition of IL2 was shown to increase the activity of total cellular phosphatidylinositol-3 kinase. These results suggest that tyrosine phosphorylation-dependent activation of phosphatidylinositol-3 kinase is a component of the IL2-receptor signal transduction process.

H 239 TWO RECEPTORS ENCODED BY A SINGLE GENE: ANALYSIS OF THE KGF AND FGF RECEPTORS

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Keratinocyte growth factor (KGF) is a new member of the fibroblast growth factor (FGF) family [Finch, *et al.*, Science *245*, 752-755, 1989]. This factor differs from other characterized FGF-related molecules which are active on a broad range of cell types, in that the mitogenic activity of KGF is tightly restricted to epithelial cells. We isolated the KGF receptor (KGFR) cDNA using a new mammalian expression vector [Miki *et al.*, PNAS *88*, 5167-5171, 1991] by means of an expression cloning strategy, in which a cDNA library from mouse BALB/MK keratinocytes was used to transfect NIH/3T3 fibroblasts. Since NIH/3T3 cells secrete KGF and BALB/MK cells express KGFR, it was possible to identify KGFR cDNA transfectants as transformed foci, in which introduction of the KGFR cDNA created an autocrine KGF transforming loop [Miki *et al.*, Science *251*, 72-75, 1991].

We recently isolated the human KGFR from B5/589 human mammary epithelial cell cDNA library using the same strategy. Structural analysis of the receptor cDNA revealed identity with one of the FGF receptors (FGFR-2) encoded by the *bek* gene, except for a divergent stretch of 49 amino acids in their extracellular domains. Binding assays demonstrated that the KGFR was a high affinity receptor for both KGF and acidic FGF, while FGFR-2 showed high affinity for basic FGF and acidic FGF but no detectable binding by KGF. Structural analysis of the human *bek* gene revealed two alternative exons responsible for the region of divergence between the two receptors. The KGFR transcript was specific to epithelial cells, and it appeared to be differentially regulated with respect to the alternative FGFR-2 transcript.

H 240 DEFECTIVE POST-TRANSLATIONAL PROCESSING ACTIVATES THE TYROSINE KINASE ENCODED BY THE *MET*/HGF RECEPTOR PROTO-ONCOGENE, Anna Mondino, Silvia Giordano, and Paolo M. Comoglio. Department of Biomedical Sciences & Oncology. University of Torino Medical School. 10126 Torino. Italy.

The *MET* proto-oncogene encodes a 190 kDa disulphide-linked heterodimeric receptor ($p190^{\alpha\beta}$) whose tyrosine kinase activity is triggered by Hepatocyte Growth Factor (HGF). The mature receptor is made of two subunits: an α chain of 50 kDa and a β chain of 145 kDa, arising from proteolytic cleavage of a single chain precursor of 170 kDa (pr170). In a colon carcinoma cell line the precursor is not cleaved and the *Met* protein is exposed at the cell surface as a single chain polypeptide of 190 kDa ($p190^{NC}$). The expression of the uncleaved *Met* protein is due to a defective post-translational processing since in this cell line: (1) the proteolytic cleavage site Lys_{303}-Arg-Lys-Lys-Arg-Ser_{308} is present in the precursor; (2) $p190^{NC}$ is sensitive to mild trypsin digestion of the cell surface, generating α and β chains of the correct size; (3) the 205 kDa insulin receptor precursor is not cleaved as well. $P190^{NC}$ is a functional tyrosine kinase *in vitro* and is activated *in vivo*, as shown by constitutive autophosphorylation on tyrosine. The *MET* gene is neither amplified nor rearranged in the colon carcinoma cell line. Overlapping clones selected from a cDNA library, derived from the mRNA of this cell line, were sequenced. No mutations were present in the *MET* coding region. These data indicates that the tyrosine kinase encoded by the *MET* proto-oncogene can be activated as consequence of a post-translational defect.

H 241 LIGAND-INDUCED POLYUBIQUITINATION OF THE PLATELET-DERIVED GROWTH FACTOR β-RECEPTOR

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We have analyzed the nature of ligand-induced shift to higher molecular weight forms of the β-receptor for platelet-derived growth factor (PDGF) expressed in porcine aortic endothelial cells. The modification of the β-receptor was found to be due to poly-ubiquitination, as judged by immunoblotting using an anti-ubiquitin antiserum. A mutant β-receptor made kinase negative by a point mutation (K634A mutant), did not undergo ubiquitination in response to ligand stimulation. A mutant in which C-terminal 98 amino acids were deleted (CT98 mutant), and which retained kinase activity, was likewise not ubiquitinated. These data suggest that the kinase activity, as well as the C-terminal 98 amino acids, is required for ubiquitination of the β-receptor. In spite of the same efficiency of ligand-induced internalization of the receptor as the wild-type β-receptor, ligand-induced degradation of the receptor-bound ligand, as well as of the receptor itself, was partially impaired in the CT98 mutant-receptor-expressing cells. These data suggest that the ubiquitination is involved in the intracellular transport or sorting of the ligand-receptor complex, or is of importance for efficient degradation of the complex. It is conceivable that the efficient removal of the ligand-stimulated PDGF β-receptor from the inside of the cells, possibly involving receptor ubiquitination, is an important mechanism for control of growth stimulation.

References: Mori, S., et al., 1991, J. Biol. Chem., in press. Mori, S., et al., EMBO J., submitted.

H 242 EGF Receptor Regulation by Threonines 654 and 669 Morrison, P#., Takishima, K.+ and Rosner, M.R.# *#The University of Chicago, The Ben May Institute, 5841 S. Maryland Ave., Box 424, Chicago, IL 60637. +First Deparment of Biochemistry, National Defense Medical College, Saitama, Japan.*

Although the regulation of the epidermal growth factor (EGF) receptor has been extensively studied, the precise role of phosphorylation at threonines 654 and 669 is not completely understood. Numerous studies have established that threonine 654 is a target for phosphorylation and down regulation by protein kinase C. No significant biological role has been identified for phosphorylation of threonine 669, the major site of EGF receptor phosphorylation. Through the placement of a negatively-charged amino acid structurally similar to phosphothreonine at these two sites, the function of their phosphorylation can be studied. In this study, we have mutated the threonine residues of the EGF receptor at sites 654 and/or 669 to glutamic acid and transfected the wild type and mutant receptor cDNAs into CHO cells, which lack endogenous EGF receptors. The EGF binding properties of the mutant receptors were, in general, similar to those of the wildtype receptors. The mutant receptors all had EGF-stimulated tyrosine kinase activity, and EGF stimulated DNA synthesis in all cases. In contrast to EGF binding, the response of the mutant receptors to phorbol ester treatment differed dependent upon the particular set of altered residues. These results suggest a role for threonine 669 in affecting the EGF receptor kinase activity as well as implicating this residue along with threonine 654 in regulating receptor signalling.

H 243 SIGNAL TRANSDUCTION THROUGH THE HUMAN LOW AFFINITY IgG RECEPTOR (FcγRIIIA), Monica S. Murakami, Urs Wirthmueller, Tomohiro Kurosaki, Jeffery V. Ravetch, Program in Molecular Biology, Sloan-Kettering Institute for Cancer Research, Cornell Graduate School of Medical Sciences, New York, NY 10021.

The constant region (Fc) of an IgG immunoglobulin is recognized by a family of transmembrane receptors. These receptors (FcγR) are members of a complex multigene family and are classified into three groups based on structural differences: FcγRI, FcγRII and FcγRIII. Each class of Fc receptor displays different cell type expression patterns. Unfortunately, the expression patterns often overlap such that no one cell type expresses only one class of Fc receptor (with the exception of the natural killer cell which expresses FcγRIIIA exclusively). In order to study each receptor type separately, we have developed a heterologous transfection system containing the necessary components sufficient to permit proper signal transduction. Using a Jurkat T-cell line it was possible to reconstitute intact signalling (Wirthmueller, U., Kurosaki, T., Murakami, M. and Ravetch J. V., manuscript in preparation). Stable Jurkat T-cell lines expressing the FcγRIIIA α and γ subunits were isolated. When these cells are specifically stimulated by the mouse monoclonal antibody 3G8 (which recognizes the extracellular domain of the FcγRIII A α subunit), we observe stimulation of PIP_2 hydrolysis, calcium influx , IL-2 production and tyrosine phosphorylation. Using this transfected cell system we hope to define the components involved in the signal transduction pathway of FcγRIIIA activation.

H 244 NERVE GROWTH FACTOR BINDS TO THE 140 KD *trk* PROTO-ONCOGENE PRODUCT AND STIMULATES ITS ASSOCIATION WITH THE *src* HOMOLOGY DOMAIN OF PHOSPHOLIPASE C γ1, Masahide Ohmichi*†, Stuart J. Decker#†, Long Pang*†, Kim L. Milarski†, and Alan R. Saltiel*†, Departments of *Physiology and #Microbiology, University of Michigan School of Medicine, Ann Arbor, MI 48109, †Department of Signal Transduction, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, MI 48105.

The cellular actions of nerve growth factor (NGF) involve regulation of protein phosphorylation. In PC-12 pheochromocytoma cells, exposure of [^{125}I]NGF followed by crosslinking indicates that the ligand binds to two discreet receptors, the previously described 75 kd protein, as well as the *trk* proto-oncogene product pp140$^{c\text{-}trk}$. Competition experiments reveal that of the two, pp140$^{c\text{-}trk}$ binds to NGF with higher affinity. Following exposure to NGF, pp140$^{c\text{-}trk}$ undergoes a rapid autophosphorylation on tyrosine residues, and concomitantly phosphorylates and associates with phospholipase Cγ1 (PLCγ1), through interaction with its *src* homology domains. The binding of NGF to pp140$^{c\text{-}trk}$ with high affinity, the NGF-dependent activation of its tyrosine kinase activity and the specific association with the effector molecule, PLCγ1, suggests that this is the biologically relevant signaling receptor for NGF. The importance of interactions between pp140$^{c\text{-}trk}$ and proteins containing *src* homology domains will be discussed.

H 245 BOMBESIN STIMULATES THE ACTIVITY OF THE MITOGEN ACTIVATED PROTEIN (MAP) KINASE IN SWISS 3T3 FIBROBLAST CELLS, Long Pang*†, Stuart J. Decker#†, and Alan R. Saltiel*†, Departments of *Physiology and #Microbiology, University of Michigan School of Medicine, Ann Arbor, MI 48109, †Department of Signal Transduction, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Company, Ann Arbor, MI 48105.

Bombesin is a potent mitogen in Swiss 3T3 cells. Although the mechanisms by which this peptide controls cellular growth remain unknown, its receptor belongs to the rhodopsin receptor family, and coupling to a G protein is involved in intracellular signaling. Because exposure of cells to numerous growth factors results in the stimulation of MAP kinase, we explored the regulation of this enzyme by bombesin. Exposure of Swiss 3T3 cells to bombesin maximally activates MAP kinase within 1 min. This stimulation is transient, and rapidly returns to basal levels. Exposure of these cells to the protein kinase C activator tetradecanoylphorbol acetate (TPA) generates an identical time course of MAP kinase activation. Down regulation of cellular protein kinase C by prolonged exposure to TPA completely abolishes the stimulatory effects of bombesin, indicating a role for kinase C in mediating this action of bombesin. These results provide further evidence for discreet pathways involving both tyrosine kinase-initiated signals and G protein-initiated signals in the regulation of MAP kinase.

H 246 PHYSICAL ASSOCIATION BETWEEN THE BOVINE PAPILLOMAVIRUS E5 TRANSFORMING PROTEIN AND THE PDGF ß RECEPTOR IN TRANSFORMED CELLS, Lisa Petti, Laura Nilson, Daniel DiMaio, Department of Genetics, Yale University School of Medicine, New Haven, CT 06510.

The E5 gene of bovine papillomavirus (BPV) encodes a 44-amino acid membrane associated protein capable of inducing tumorigenic transformation in rodent fibroblast cell lines. Genetic studies suggest that the E5 protein transforms cells by influencing the activity of a cellular protein involved in growth control. Previously, we have shown that the endogenous cellular ß receptor for platelet-derived growth factor (PDGF) is constitutively activated in E5-transformed cells. We now show that the E5 protein and the activated PDGF receptor exist in a stable complex in transformed mouse C127 cells. Antibodies raised against the PDGF receptor coimmunoprecipitate the E5 protein from transformed cells. Conversely, antibodies raised against the E5 protein coimmunoprecipitate the PDGF receptor from cells transformed by the E5 protein but not from normal cells or cells transformed by v-sis. Intracellular, tyrosine phosphorylated PDGF receptor is the primary receptor species present in E5 immune complexes. Therefore, either the E5 protein and the PDGF receptor contact one another directly or both are present in a multiprotein complex. This is the first example of an association between a DNA tumor virus transforming protein and a cellular protein with a well defined role in the control of normal cell proliferation. Furthermore, we have obtained evidence that the E5 protein has little effect on mammary epithelial cells which lack PDGF receptors but that introduction of the gene encoding the PDGF ß receptor renders these cells susceptible to E5-mediated tumorigenic transformation. These results suggest that the PDGF receptor is required for transformation by E5 and thus it is an important cellular intermediate which transmits the transforming signal from the E5 protein to the cell.

H 247 THE HGF/SF TRANSDUCTOSOME: INTERACTIONS OF THE p190^{MET} RECEPTOR WITH SH2-PROTEINS. C.Ponzetto[1], A.Bardelli[1],F.Maina[1], A.Graziani[1], I.Gout[2], M.J.Fry[2], R.Dhand[2], M.D. Waterfield[2] and P.M.Comoglio[1] ([1])*Dept. of Biomedical Sciences and Oncology, University of Torino (Italy) and* ([2]) *Ludwig Institute for Cancer Research, London (England).*

The product of the *MET* proto-oncogene is the receptor for a polypeptide known as Hepatocyte Growth Factor (HGF) or Scatter Factor (SF). This molecule induces motility in several cell types and mitogenesis in others. Elucidation of the transduction pathways triggered by HGF/SF in the respective target cells could explain the dual response elicited by the ligand-receptor interaction. P190^{MET} can associate *in vitro* and *in vivo* *ras*GAP, phospholipase Cγ, p59^{FYN} and phosphatidylinositol 3-Kinase (PI 3 kinase). These associations are mediated through phosphotyrosines located at specific sites. Tyrosine phosphorylated P190^{MET} expressed in insect cells associates either recombinant p85 or the p85/p110 PI 3-kinase holoenzyme purified from bovine brain. The MET peptide EYCPDPLYEVMLK, containing the consensus sequence -YXXM- for PI 3-kinase binding, was synthesized and phosphorylated using A431 membranes. The phosphorylated peptide did bind the partially purified 85/110 PI 3-kinase complex, while the non-phosphorylated peptide did not. The role of tyrosine 1313 and 1307 in conferring the ability of binding PI 3-Kinase will be discussed on the basis of the results obtained with the respective Tyr→Phe mutants.

H 248 C-TERMINAL TRUNCATED FORMS OF THE *Met*/ HEPATOCYTE GROWTH FACTOR RECEPTOR Maria Prat, Tiziana Crepaldi, Lucia Gandino, Silvia Giordano and Paolo Comoglio Department of Biomedical Sciences and Oncology, University of Torino, School of Medicine, 10126 Torino, Italy.

The *MET* protooncogene encodes a transmembrane tyrosine kinase of 190 kDa (p190^{MET}), which has recently been identified as the receptor for Hepatocyte Growth Factor/ Scatter Factor. P190^{MET} is a heterodimer composed of two disulfide-linked chains of 50 kDa (p50$^{\alpha}$) and 145 kDa (p145$^{\beta}$). We have produced four different monoclonal antibodies, specific for the extracellular domain of the *Met* receptor. These antibodies immunoprecipitate with p190^{MET} two additional *Met* proteins of 140 kDa and 130 kDa. The first one (p140^{MET}) is membrane bound, and is composed of an α chain (p50$^{\alpha}$) and of an 85 kDa C-terminal truncated β chain (p85$^{\beta}$). The second one (p130^{MET}) is released in the culture supernatant and consists of an α chain (p50$^{\alpha}$) and of a 75 kDa C-terminal truncated β chain (p75$^{\beta}$). Both truncated forms lack the tyrosine kinase domain. P140^{MET} and p130^{MET} are consistently detected *in vivo*, together with p190^{MET}, in different cell lines or their culture supernatants. P140^{MET} is preferentially localized at the cell surface, where is present in roughly half the amount of p190^{MET}. The two C-terminal truncated forms of the *Met* receptor are also found in stable transfectants expressing the full-lenght *MET* cDNA, thus showing that they originate from post-translational proteolysis. This process is regulated by protein kinase-C activation. Altogether these data suggest that the production of the the C-terminal truncated *Met* forms may have a physiological role in modulating the *Met* receptor function.

H 249 RECEPTORS CONTROLLING DAUER LARVA DEVELOPMENT IN *C. ELEGANS*, Donald L. Riddle, Laura L. Georgi, Wen-Hui Yeh, Miguel Estevez, and Patrice S. Albert, Molecular Biology Program, University of Missouri, Columbia, MO 65211

The pathway of signal transduction that regulates development of the *C. elegans* dauer larva may be the nematode analogue of a TGF-β signalling system. The dauer larva is a non-feeding dispersal stage formed under conditions of overcrowding and limited food. When a sufficient level of a *Caenorhabditis* specific, dauer-inducing pheromone is present in the environment, specific chemosensory neurons that monitor the pheromone : food ratio allow development to proceed through a pre-dauer second-stage larva to the dauer stage. When dauer larvae encounter food in a fresh environment, they resume feeding and develop to the reproductive adult. About 25 genes have been placed in a pathway for dauer larva development based on the genetic interactions between mutant alleles. Three of these genes have been cloned by transposon-tagging, and all three encode receptor molecules. Intermediate steps in the genetic pathway are mediated by the *daf-1* and *daf-4* genes. Both these genes encode transmembrane receptor serine kinases. The *daf-1* receptor was the first such kinase reported, and the mouse activin receptor shares a number of sequence motifs in common with it. The *daf-12* gene specifies what we believe to be the last step in signal transduction. It encodes a member of the steroid/thyroid hormone receptor superfamily. Its sequence appears to be most similar to the human Vitamin D and Retinoic Acid receptors. Whereas *daf-1* and *daf-4* are required for normal non-dauer development, *daf-12* activity is required for dauer larva morphogenesis. We propose that the activin-receptor-like *daf* kinases phosphorylate proteins that promote growth, and directly or indirectly inactivate the *daf-12* receptor, possibly by preventing synthesis of a dauer-inducing hormone.

H 250 THE ALTERNATIVE SPLICING OF THE CD45 TYROSINE PHOSPHATASE IS CONTROLLED BY NEGATIVE *REGULATORY TRANS-ACTING SPLICING FACTORS.* David M. Rothstein, Haruo Saito, Michel Streuli, Stuart F. Schlossman and Chikao Morimoto, Division of Tumor Immunology, Dana-Farber Cancer Inst., Boston, MA 02115

CD45, a major cell surface glycoprotein having protein tyrosine phosphatase activity, has been shown to play an important role in T cell activation. It consists of 5 isoforms generated by the alternative mRNA splicing of three exons encoded by a single gene. The various isoforms are differentially expressed on T cells having distinct functions and may direct distinct ligand and substrate interactions. The CD45 splicing pattern is tissue-specific and also related to activation and differentiation. B cells primarily express the largest (CD45RA) isoforms. Thymocytes express the smallest (CD45RO) isoform, but upon export into (neonatal) peripheral blood, their splicing pattern changes and CD45RA is primarily produced. T cells differentially express the CD45 isoforms, and after activation, their pattern of expression changes in a highly regulated fashion. We previously showed that mouse B and thymocyte cell lines transfected with the same human mini-CD45 gene construct, appropriately splice alternative exons in or out, respectively. This demonstrated the presence of trans-acting *tissue-specific splicing factors. To study the regulation of* alternative splicing of CD45, we have developed an in vivo complementation assay. Cells having different CD45 splicing patterns were transiently fused with PEG, and CD45 mRNA splicing pattern evaluated at different time-points by RT-PCR specific for human CD45. Human B cell lines (CD45RA+) were able to produce lower m.w. splice products (CD45RO) after fusion with mouse thymocytes. On the other hand, mouse B cells were unable to alter the splicing pattern of human T cell lines (CD45RO+). This suggests that cells expressing CD45RO contain negatively acting factors that allow the alternatively spliced exons to be skipped and that the full length CD45RA isoform containing all 3 alternative exons is the default pattern of mRNA splicing. Fusion of human B cells with NIH-3T3 cells, also results in the production of the lower m.w. splice products, suggesting that cells not expressing CD45 may contain splice factors capable of regulating CD45 splicing. In agreement, incubation of thymocytes or T cell subsets with cycloheximide resulted in an increase in the larger CD45 mRNAs. We can now interpret the changes in CD45 isoform expression in terms of the presence or absence of negative regulatory trans-acting splicing factors.

H 251 ASSOCIATION OF CD4:p56^{lck} WITH THE T-CELL ANTIGEN RECEPTOR (TCR/CD3) UPREGULATES *DEFECTIVE CD3-MEDIATED EARLY SIGNAL EVENTS* IN TCR/CD3^{low} EXPRESSOR T CELLS THROUGH A PROTEIN TYROSINE KINASE-DEPENDENT MECHANISM. Jaime Sancho*, Jeffrey A. Ledbetter#, Myung-Sik Choi*, Steven B. Kanner#, Julie P. Deans# and Cox Terhorst*. *Dana-Farber Cancer Institute, Boston, MA 02115 and #Oncogen/Bristol-Myers Squibb, Seattle, WA 98121.

It has been proposed that during T cell receptor antigen recognition CD4: or CD8:p56^{lck} molecules interact with the T-cell antigen receptor-CD3 complex (TCR/CD3) to phosphorylate various undefined substrates, which then initiate signal transduction through the TCR/CD3 complex. The ability of CD4 to modulate the TCR/CD3-induced increase in intracellular calcium, $[Ca^{2+}]_i$, and substrate tyrosine phosphorylation was studied in mutants of the human leukemic T cell line HPB-ALL characterized by their low expression of the TCR/CD3 complex on the cell surface. In HPB-ALL mutants in which CD3-ζ_2 was found to be associated with the other chains of the TCR/CD3 complex on the cell surface, ligation of TCR/CD3 with CD4 resulted in a profile of calcium mobilization similar to that observed in wild-type HPB-ALL cells, despite a defective induction of calcium response and tyrosine kinase activity upon CD3 engagement. Furthermore, in these mutants the magnitude of $[Ca^{2+}]_i$ correlated well with the magnitude of tyrosine phosphorylation of PLC-γ1 and CD3-ζ but not with increases in the general pattern of substrate tyrosine phosphorylation. In contrast, in a mutant which was defective in CD3-ζ_2 association, the increments of $[Ca^{2+}]_i$ and substrate tyrosine phosphorylation induced by crosslinking CD3 and CD4 were minimal or absent respectively. These experiments showed that the presence of CD3-ζ_2 in the TCR/CD3 complex is of critical importance for the ability of CD4 to enhance early transducing signals inside the cell. The data also suggest that CD4-associated protein tyrosine kinase p56^{lck} could upregulate defective CD3-mediated induction of PLC activity by increasing tyrosine phosphorylation of PLC-γ1.

H 252 *AN INTRACYTOPLASMIC DOMAIN OF THE* BOMBESIN/GRP RECEPTOR BINDS PROTEIN KINASE ACTIVITY IN SWISS 3T3 CELL EXTRACTS. *Thomas P. Segerson, Suzanne Montgomery. Vollum Institute, Portland, OR 97201.* The mammalian counterpart of the amphibian neuropeptide bombesin, gastrin-releasing peptide (GRP), is a potent growth factor for fibroblast, lung, and gastrointestinal cells and appears to be a potent autocrine growth factor for small-cell lung carcinoma cells. The pathways utilized by GRP to produce cellular signals leading to mitogenesis in mammalian cells are poorly characterized. Although GRP receptor stimulation clearly activates PI hydrolysis, additional mechanisms such as protein tyrosine phosphorylation may contribute to the effects of GRP on cell growth. GRP receptor activation is associated with the activation of a tyrosine kinase activity. Some evidence suggests that the GRP receptor associates physically with a protein tyrosine kinase. We have used a fusion protein of the carboxy-terminal intracellular portion of the GRP receptor fused to *S. japonicum* glutathione-S-transferase and a glutathione affinity column to identify the proteins in Swiss 3T3 cells that bind to this region of the receptor molecule. Swiss 3T3 cell proteins were labeled by the addition of [^{35}S]methionine to the culture for four hours. Cell lysates was pre-cleared with an affinity matrix of the *S. japonicum* glutathione-S-transferase protein alone and then incubated for with the GRP receptor fragment fusion protein affinity matrix. The beads were collected by centrifugation and the proteins were eluted by boiling in Laemmli buffer, followed by SDS-PAGE fractionation and autoradiography. At least four proteins from Swiss 3T3 cell extracts appear to bind specifically to the GRP receptor affinity matrix. To determine whether any of the proteins that bind to the GRP receptor fragment represent functional kinases, lysates from unlabeled Swiss 3T3 cells treated identically and bound proteins were rinsed twice with lysis buffer and resuspended in 40 µl of 10 mM $MnCl_2$ /50 mM Tris pH 7.4, and 20 µCi [γ^{32}P]ATP followed by a 20 min incubation at room temperature. Proteins were analyzed by SDS-PAGE and autoradiography. Phosphorylation of proteins that were bound to the GRP receptor fragment, including the GRP receptor fragment itself, was detected, whereas there was negligible phosphorylation of proteins that bound to the glutathione-S-transferase protein. This indicates that at least one of the proteins that binds to the GRP receptor fragment is a protein kinase and that, at least *in vitro*, the GRP receptor fusion protein is a substrate for this kinase. We will now determine whether the GRP fragment itself (minus fusion sequence) is a substrate and whether this represents a tyrosine or serine/threonine kinase activity, or both.

H 253 CHARACTERIZATION OF MONOCLONAL ANTIBODIES REACTIVE WITH THE EXTRACELLULAR DOMAIN OF THE c-erbB-2 PROTEIN AND THEIR INTERACTION WITH CIS-DIAMMINEDICHLORO-PLATINUM (CDDP), Laura K. Shawver, Susan S. Elliger, and Beatrice C. Langton, Department of Cell Biology and Immunology, Berlex Biosciences, Alameda, CA 94501.

The c-erbB-2 gene encodes a 185 kDa tyrosine kinase receptor (185) which, when overexpressed, has prognostic significance for breast and ovarian cancer. We have recently shown that a monoclonal antibody (TAb 250) recognizing an extracellular determinant of p185 interacts with CDDP in a synergistic manner to inhibit proliferation of tumor cells expressing this protein (Hancock et al., Cancer Res. 51:4575, 1991). We have also found that TAb 250 shares several properties of a growth factor including phosphorylation and down-modulation of the receptor protein, and internalization of the antibody molecule (Shawver et al., J. Cell. Biochem. Suppl. 15B:113, 1991). In order to determine if ligand-like properties are important for interaction with CDDP, additional antibodies previously shown to bind the extracellular domain of p185 (Langton et al., Cancer Res. 51:2593, 1991) were characterized. We examined their ability to compete with ^{125}I-TAb 250 for binding, ability to stimulate phosphorylation of the c-erbB-2 protein in an in vitro autokinase assay, and ability to inhibit tumor cell growth in vitro and in vivo. Four antibodies with different properties were chosen for further study. TAb 257 was found to be similar to TAb 250 in that it competed with ^{125}I-TAb 250 for binding and also stimulated kinase activity. TAb 255 was able to compete with ^{125}I-TAb 250 for binding, but did not stimulate kinase activity. Neither TAb 260 nor 263 could displace ^{125}I-TAb 250 binding; however, TAb 263 stimulated kinase activity while TAb 260 did not. In an in vitro cellular proliferation assay, TAbs 255 and 257 showed similar anti-proliferative activity as TAb 250 while TAb 263 was less effective. We will report on the interaction of these antibodies with cisplatin in vivo using a subcutaneous xenograft model in athymic mice, and the potential implications of these findings for antibody/drug combination therapy.

H 254 TYROSINE PHOSPHORYLATION AND RECEPTOR-TRIGGERED T CELL ACTIVATION, Jia Shi and Richard A. Miller, Dept. of Pathology, Boston Univ. Sch. of Med. Boston, MA 02118; Inst. of Gerontology, Univ. of Michigan, Ann Arbor, MI 48109.

Antiphosphotyrosine immunoblots were used to characterize tyrosine phosphorylated proteins after stimulation of the murine T lymphocyte antigen receptor (TCR). Increased tyrosine phosphorylation was evident on at least 4 substrates as early as 2 min after addition of anti-TCR monoclonal antibodies or after cross-linking the TCR with its co-receptor CD4 molecule. Of interest is that increasing phosphorylation of the 110 kD and 80 kD proteins by anti-CD3 antibody treatment is preferentially diminished in T cells from old (20-22 mo) mice. These findings show, in agreement with others, that increased tyrosine phosphorylation is one of the earliest signals after the TCR complex is bound, and they reveal that there are specific age-dependent impairments of tyrosine phosphorylation upon TCR mediated stimulation.

H 255 SIGNAL TRANSDUCTION THROUGH THE T CELL RECEPTOR FOR ANTIGEN. J. Siegel, C. June, U. Rapp and L. Samelson, Naval Medical Research Institute, Bethesda, MD: National Cancer Institute, Frederick MD; and the Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda MD. The T cell receptor for antigen (TCR) is a multichain complex on the surface of T lymphocytes which recognizes specific antigen and initiates a signal transduction cascade ultimately leading to lymphokine production and cell proliferation. When the TCR is engaged, two signal transduction pathways are initiated: a tyrosine kinase is activated leading to phosphorylation of a variety of substrates; and phospholipase C (PLC) is activated leading to increased turnover of phosphatidylinositol (PI) and consequent production of IP_3 and diacylglycerol (DAG). These latter products lead to increases in intracellular calcium and activation of protein kinase C (PKC). In order to investigate downstream signaling events, we have examined the role of the cytosolic serine/threonine kinase c-raf in TCR-mediated signal transduction. We have previously found that in murine T cell hybridomas the c-raf kinase is activated upon TCR ligation by a PKC dependent mechanism, demonstrating that c-raf is part of the PLC pathway. When we examined human peripheral blood T cells, we found that CD3 ligation was a very efficient stimulus for c-raf activation as was crosslinking of CD2 and CD4 but not CD28 or CD45. Time course experiments demonstrated extremely early kinetics with c-raf activation observed as early as 30 sec in peripheral blood T cells and by 15 sec in the Jurkat T cell line. These data indicate that c-raf activation is one of the earliest documented TCR signaling events. Nonetheless, comparative kinetic experiments showed that activation of c-raf was preceded by activation of the tyrosine kinase and PLC as evidenced by increases in intracellular calcium and IP_3. Thus, coupling of the TCR to PKC activation and to downstream signaling events occurs rapidly and efficiently in human T lymphocytes. Subsequent studies will show whether c-raf mediates new gene transcription in response to TCR engagement as it does in the response to growth factors.

H 256 BIOCHEMICAL CHARACTERIZATION OF A CLONED MOUSE PHOSPHOTYROSINE PHOSPHATASE (MPTPASE) Ulrich Tillmann, Michel L. Tremblay, Bedrich Mosinger Jr.and Heiner Westphal
Laboratory of Mammalian Genes and Development, National Institutes of Health, Bethesda, Md. 20892

A mouse cDNA encoding a non-receptor-type phosphotyrosine phosphatase (MPTPase) has been isolated that is highly homologous to a previously described human T-cell PTPase (TCPTPase). The MPTP gene is transcribed in a variety of tissues with marked enhancement of expression in thymus, testis, ovary and kidney. Translation of the single open reading frame in bacteria yields an enzyme activity that is specifically inhibited by sodium orthovanadate. Anti-peptide antibodies and antisera raised against a Glutathione-S-Transferase-MPTPase fusion protein were used to immunoprecipitate the enzyme from available testis and kidney carcinoma cell lines. Immunofluorescence studies on these cell lines have localized the MPTPase at the cytoskeleton. It is presently unclear how the sequestration to the cytoskeleton is achieved and which phosphoproteins are the in vivo substrates for this MPTPase. Starting out with a biochemical characterization of MPTPase expressed in bacteria we will present experiments aimed at clarifying the questions raised above.

H 257 HUMAN MALIGNANT MESOTHELIOMA CELL LINES EXPRESS PDGF β-RECEPTORS WHEREAS CULTURED NORMAL MESOTHELIAL CELLS EXPRESS PREDOMINANTLY PDGF α-RECEPTORS *M.A. Versnel[1], L. Claesson-Welsh[5], A. Hammacher[5], M.J. Bouts[1], Th.H. van der Kwast[2], A. Eriksson[5], H.C. Hoogsteden[3], A. Hagemeijer[4] and C.-H. Heldin[5]*
[1]Departments of Immunology, [2]Pathology, [3]Pulmonary Medicine and [4]Cell Biology and Genetics, Erasmus University and Academic Hospital Dijkzigt, Rotterdam, The Netherlands and [5]Ludwig Institute for Cancer Research, Uppsala, Sweden

In human malignant mesothelioma cell lines elevated expression of the PDGF B-chain (c-*sis*) gene was previously reported, while normal mesothelial cells barely express this gene. Expression of the PDGF A-chain gene was only slightly elevated in these cell lines compared to normal mesothelial cells. For a putative autocrine function of the produced PDGF, in these cells expression of PDGF receptors is a prerequisite. Here, we report on the expression of PDGF α- and β-receptors in normal and malignant mesothelial cells. Cultured normal mesothelial cells expressed PDGF α-receptor mRNA and protein and had weak levels of the PDGF β-receptor mRNA and protein. In contrast, malignant mesothelioma cell lines were found to express PDGF β-receptor mRNA and protein, while PDGF α-receptor expression was not detectable by Northern blotting and immunoprecipitation. Binding experiments with ^{125}I-PDGF-AA and ^{125}I-PDGF-BB to normal and malignant mesothelial cell lines confirmed these observations. These results suggest that autocrine stimulation of growth may occur both in cultured normal mesothelial cells (PDGF-AA acting via the α-receptor) and in malignant mesothelioma cell lines (PDGF-BB acting via the β-receptor).

H 258 HGF-SF/Met RECEPTOR ASSOCIATES A PROTEIN TYROSINE PHOSPHATASE, Emma Villa-Moruzzi*, Simone Lapi*, Nicholas K. Tonks+, Giovanni Gaudino# and Paolo M. Comoglio#, *Dept. Biomedicine, Univ. of Pisa, 56126 Pisa, Italy, +Cold Spring Harbor Lab., Cold Spring Harbor, NY 11724, #Dept. Biomedical Sciences & Oncology, Univ. of Torino, 10126 Torino, Italy.

The receptor for Hepatocyte Growth Factor, also known as Scatter Factor (HGF-SF) has been identified as the 190 kDa heterodimeric tyrosine kinase encoded by the MET protooncogene (p190MET). In the gastric carcinoma cell line GTL-16 MET is amplified, overexpressed and the tyrosine kinase is activated. In this and other cell lines examined over 90% of the protein tyrosine phosphatase (PTP) activity is associated with the particulate fraction (extracted by Triton X-100/NaCl). In FPLC gel filtration of this fraction Met (identified by Western analysis) and PTP activity (assayed with P-Tyr myelin basic protein) co-eluted in a high molecular weight fraction. In immunoprecipitates made from GTL-16 cells using specific monoclonal antibodies directed against the extracellular domain of Met, PTP activity as well as tyrosine kinase activity of p190MET were present. This PTP activity was able to dephosphorylate the "in vitro" autophosphorylated Met. Activation of PKC in intact GTL-16 cells by TPA negatively regulated the tyrosine kinase activity detected in the Met immunoprecipitate and decreased its associated PTP activity. When Met immunoprecipitates were analyzed by immunoblotting with an antibody raised against the catalytic domain of the low Mr T cell PTP, a protein of approximately 55 kDa was detected. In anti-Met immunoprecipitates from TPA-treated cells this protein was absent. This implies that the decrease in PTP activity observed upon TPA treatment may be due to the dissociation of the 55 kDa species. These data suggest a specific interaction between p190MET and a PTP associated with the particulate cellular fraction.

H 259 ACTIVATION OF MAP KINASES BY MULTIPLE HEMOPOIETIC GROWTH FACTORS. Melanie J. Welham, Vince Duronio, Jasbinder S. Sanghera, Steven L. Pelech and John W. Schrader. The Biomedical Research Centre, 2222 Health Sciences Mall, U.B.C., Vancouver, British Columbia, V6T 1Z3, Canada.

Stimulation of hemopoietic cells with IL-3, IL-4, IL-5, GM-CSF and *Steel* factor (SLF), induces tyrosine phosphorylation of a number of protein substrates. Two of these proteins, designated p42 and p44, are tyrosine phosphorylated rapidly in response to treatment with IL-3, IL-5, GM-CSF and SLF, but not IL-4. We have demonstrated that these common substrates are members of the MAP kinase family of serine/threonine protein kinases, characterised by their ability to phosphorylate myelin basic protein (MBP). Ion-exchange chromatography was used to partially purify MAP kinases from extracts prepared from stimulated and unstimulated cells. Kinase assays and immunoblotting of column fractions demonstrated co-elution of the peak of MAP kinase enzyme activity with the p42 and p44 tyrosine phosphorylated species, and with two proteins of 42 and 44 kDa which were immunoreactive with anti MAP kinase antisera. Time-course analyses demonstrated *Steel* factor activation of MAP kinase activity was maximal after 2 minutes of factor treatment and decreased to basel levels within 30 minutes of stimulation. Interestingly, activation of MAP kinase after IL-5 treatment was not so rapid. Maximal activity was observed 15 minutes after stimulation and remained elevated for up to 60 minutes following IL-5 addition. Comparison of the substrate specificty of the enzymes activated by SLF and IL-5 suggest these two factors may be activating different isoforms of MAP kinase. These results demonstrate that members of the MAP kinase family, known to be activated by multiple agents, are involved in common signal transduction events elicited by IL-3, IL-5, GM-CSF and *Steel* factor and therefore represent an important point of convergence of signalling pathways in hemopoietic cells.

H 260 Identification and Characterization of a Novel Receptor Protein Tyrosine Kinase, Tai Wai Wong and Xueyu Shen, Dept. of Biochemistry, Robert Wood Johnson Medical School, Piscataway, N. J. 08854

We have identified a human cDNA that encodes a novel protein tyrosine kinase. The deduced amino acid sequence exhibits features that are characteristic of receptor type kinases. The corresponding mRNA, which is about 6.5 kb in size, is expressed in almost all of the major tissues and in a variety of cell lines. The cDNA has been expressed in bacteria and the protein product has been used to generate polyclonal antibodies. Using the antibodies, we have identified the protein product in tissue culture cells. We will present results of characterization of the kinase activity, its subcellular distribution, and activation of the kinase activity.

H 261 STIMULATION OF TYROSINE KINASE ACTIVITY IN ANTI-PHOSPHOTYROSINE IMMUNE COMPLEXES OF SWISS 3T3 CELL LYSATES OCCURS RAPIDLY AFTER ADDITION OF BOMBESIN, VASOPRESSIN, AND ENDOTHELIN TO INTACT CELLS, Ian Zachary, James Sinnett-Smith and Enrique Rozengurt, Imperial Cancer Research Fund, P.O.Box 123, Lincoln's Inn Fields, London WC2A 3PX, U.K.

Bombesin, vasopressin and endothelin rapidly increase tyrosine and serine phosphorylation of multiple substrates in quiescent Swiss 3T3 cells. To determine whether neuropeptide stimulation of tyrosine phosphorylation was due to activation of a protein tyrosine kinase, kinase activity was measured in anti-phosphotyrosine immunoprecipitates prepared from neuropeptide-treated cells. Treatment of quiescent Swiss 3T3 cells with bombesin, vasopressin, endothelin/vasoactive intestinal contractor (VIC), and bradykinin strikingly increased the initial rate of tyrosine phosphorylation measured in anti-phosphotyrosine immunoprecipitates, of a major band of Mr 115,000 (p115) and two minor components of Mr 90,000 and 75,000. Neuropeptides increased the labelling of p115 within seconds and with great potency; half-maximum concentrations were 0.1, 0.2 and 0.3 nM for bombesin, vasopressin and VIC, respectively. Immunoblotting and peptide mapping showed that the p115 band phosphorylated in anti-phosphotyrosine immunoprecipitates is identical to a major Mr 115,000 substrate for neuropeptide-stimulated tyrosine phosphorylation in intact Swiss 3T3 cells. Furthermore, bombesin, vasopressin and VIC markedly increased the rate of phosphorylation of Raytide, a broad-specificity tyrosine kinase peptide substrate, by decreasing (8 ± 1.3-fold) the apparent Km of the kinase for the substrate. Phorbol 12,13 dibutyrate and the Ca^{2+} ionophore A23187 had a weaker effect on tyrosine protein kinase activity in immune complexes compared to bombesin. Furthermore, down-regulation of protein kinase C blocked the small effect of phorbol esters, but did not impair bombesin-stimulated tyrosine kinase activity. These results provide direct evidence for neuropeptide activation of a tyrosine kinase in cell-free preparations and identify a novel event in the action of this class of growth factors in Swiss 3T3 cells.

H 262 STUDIES OF CHIMERIC RECEPTORS CONSTRUCTED FROM INSULIN RECEPTOR AND INSULIN RECEPTOR-RELATED RECEPTOR, B. Zhang, A. Suzuki*, A.B. Smit#, and R.A. Roth, Department of Pharmacology, Stanford University School of Medicine, Stanford, CA 94305, *Department of Agricultural Chemistry, University of Tokyo, Bunkyo-Ku, Tokyo 113, Japan, #Biological Laboratory, Vrije Universiteit, PO Box 7161, 1007 MC Amsterdam, The Netherlands

The gene for a new receptor, the insulin receptor-related receptor (IRR), was isolated by Shier and Watt by screening of genomic libraries with the insulin receptor (IR) cDNA. The ligand recognized by IRR, its tissue distribution, and its signaling pathways are all unknown. In an attempt to address these issues, we have constructed a chimeric receptor with exons 2 and 3 of IR replaced with the comparable exons of IRR, since earlier studies have shown this region of IR is responsible for high-affinity ligand binding. This chimeric receptor was able to bind ten conformation-dependent monoclonal antibodies to the extracellular domain, was processed into mature α and β subunits, and its tyrosine kinase activity was activated by an insulin mimetic monoclonal antibody (83-14). However, this chimera did not bind radioactively labeled insulin, IGF-I, IGF-II, relaxin, bombyxin II, and bombyxin IV. These ligands and molluscan insulin-related peptide (MIP) did not stimulate the kinase activity of the chimera as measured by Western blots with anti-phosphotyrosine antibodies. To determine the expression of IRR mRNA, PCR was performed using RNA from rat tissues and various cell lines. The IRR mRNA was identified in both rat and human kidneys, but not in any of the cell lines studied. In conclusion, IRR does not appear to be the receptor for any known insulin-related ligand, but it does appear to be expressed in kidney.

H 263 FUNCTIONAL AND COMPARATIVE ANALYSIS OF THE CYTOPLASMIC DOMAIN OF THE HUMAN GRANULOCYTE COLONY-STIMULATING FACTOR RECEPTOR, Steven F. Ziegler, David P. Gearing, Bruce Mosley, Jefferey A. Schneringer, Douglas E. Williams and Craig A. Smith. Immunex Corp. Seattle, WA 98101.

Two cDNAs encoding the human G-CSF receptor, which differ only in the cytoplasmic domain, have previously been isolated from a human placental cDNA library (Larsen *et al.*, 1990. J. Exp. Med. **172:** 1559). An analysis of the human G-CSF-R gene showed that the difference was due to differential RNA splicing of a cryptic intron within the coding region of the gene. Expression of either cDNA in the murine IL-6-dependent line B9 or the murine IL-3-dependent line BAF/BO3 conferred G-CSF-responsiveness. Deletional analysis of the cytoplasmic domain of the human G-CSF-R has revealed a region required for signal transduction. The region is contained within the first 60 amino acids of the cytoplasmic domain and is homologous to the cytoplasmic domains of the IL-6-R subunit gp130 and the recently cloned LIF-R (Gearing *et al.*, EMBO J., in press). To further analyze the function of these sequences, we have constructed a number of chimeric molecules using the extracellular and cytoplasmic domains of these three receptors. These chimeric receptors have been expressed in BAF/BO3 cells in order to study the functional relationships between these receptors.

Cytoplasmic Signalling Molecules

H 300 A TPA INDUCIBLE INCREASE IN ACTIVITY OF A MAP KINASE ACTIVATOR, Peter D. Adams and Peter J. Parker, Protein Phosphorylation Department, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX.

Threonine and tyrosine residue phosphorylation of a 42 kDa protein identified as mitogen activated protein kinase (MAP kinase)[1] is shown to be stimulated in extracts from TPA pretreated U937 cells. This is accompanied by an increase in MAP kinase activity. It is further demonstrated that TPA pretreatment leads to the induction of an activity that will reactivate dephosphorylated/inactivated MAP kinase. This TPA-induced activity induces the threonine and tyrosine phosphorylation of a 42 kDa protein in extracts from unstimulated cells[2].

1 L.B. Ray and T.W. Sturgill (1988) J. Biol. Chem. 263, 12721-12727.
2 P.D. Adams and P.J. Parker (1991) FEBS Letters, in press.

H 301 *Ras* GTPase ACTIVATING PROTEIN (GAP): A POTENTIAL LINK BETWEEN PROTEIN TYROSINE KINASES AND G-PROTEINS IN T LYMPHOCYTES, Kurt E. Amrein, Nicholas Flint, Bärbel Panholzer, Erich Küng, Paula Armbruster and Paul Burn, Department of Biology, PRT, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland

Ras GTPase activating protein (GAP) is a cytoplasmic factor that regulates the GTPase activity of $p21^{ras}$. Phosphorylation of GAP on tyrosine has recently been reported by several groups and may be an important step in linking signalling pathways involving $p21^{ras}$ and protein tyrosine kinases. In T lymphocytes both, PTKs ($p56^{lck}$ and $p59^{fyn}$) and G-proteins, have been proposed to be involved in signal transduction pathways and T cell activation. To investigate whether GAP is a substrate of $p56^{lck}$ and whether in T lymphocytes a link exists between PTKs and G-proteins we performed phosphorylation studies using purified, recombinant human GAP (kindly provided by Dr. A. Wood et al., Roche Nutley) and purified, recombinant human $p56^{lck}$. In a first series of experiments we used GAP and $p56^{lck}$ in *in vitro* kinase reaction experiments. Our experiments demonstrated that GAP protein can serve as an *in vitro* substrate for $p56^{lck}$. Tryptic peptide map and phosphoaminoacids analysis of *in vitro*-phosphorylated GAP revealed one major labeled phosphopeptide containing phosphotyrosine. We identified the tryptic phosphopeptide by comigration on 2D maps with in vitro synthesized, candidate phosphopeptides. Thus, although GAP contains 38 tyrosine residues, it seems to be phosphorylated selectively by the purified $p56^{lck}$ on one particular tyrosine residue. In a second series of experiments we performed co-immunoprecipitation studies from *in vitro* kinase reaction mixtures containing recombinant $p56^{lck}$ and GAP using anti-$p56^{lck}$ antiserum as a probe. By this means we demonstrated that $p56^{lck}$ is bound preferentially to GAP phosphorylated on a tyrosine residue. In summary the experiments described suggest that GAP might be a substrate of $p56^{lck}$ *in vivo*, and that in T lymphocytes the function of GAP might be regulated through its phosphorylation on tyrosine and binding to the PTK $p56^{lck}$.

H 302 TYROSINE PHOSPHORYLATION IN VIVO BY pp56^{lck} REQUIRES POLYAMINES. Leif C. Andersson, Christina Oetken, Tiina Pessa-Morikawa, Anders Ståhls, Amon Altman* and Tomas Mustelin, Department of Pathology, University of Helsinki, 00290 Finland, *Division of Cell Biology, La Jolla Institute for Allergy and Immunology, La Jolla, CA

Mitogenic stimulation of resting T lymphocytes induces rapid tyrosine phosphorylation of key regulatory proteins. The nonreceptor tyrosine protein kinases pp56^{lck} and pp59^{fyn} have been reported to participate in this pathway. Activation of T cells is also associated with a large increase in the activity of ornithine decarboxylase (ODC), the rate-limiting enzyme in the generation of the polyamines, putrescine, spermidine and spermine. The physiological role of the polyamines have, however, remained unclear. We have found that tyrosine phosphorylation by pp56^{lck} in LSTRA cells depends on an intact polyamine synthesis. Treatment of the cells with α-difluoromethylornithine (DFMO), an irreversible inhibitor of ODC, reduced the amount of phosphotyrosine in several cellular substrates, including phospholipase Cγ1(PLCγ1) and pp56^{lck}. Addition of putrescine reversed the effect of DFMO on tyrosine phosphorylation. DFMO or polyamines did not directly interfere with the catalytic activities of pp56^{lck} or tyrosine phosphatases *in vitro*. *In vivo* treatment with DFMO, however, largely inhibited the co-precipitability of pp56^{lck} and PLCγ1 while this complex was obtained after addition of putrescine to the cultures. These findings indicate that substrate binding and tyrosine phosphorylation by pp56^{lck} depends on polyamines.

H 303 TYROSINE PHOSPHORYLATED EGFR AND CELLULAR pp130 PROVIDE HIGH AFFINITY BINDING SUBSTRATES FOR THE STUDY OF CRK-PHOSPHOTYROSINE INTERACTIONS IN VITRO. Raymond B.Birge, J.Eduardo Fajardo, Bruce J.Mayer, and Hidesaburo Hanafusa. Laboratory of Molecular Oncology, The Rockefeller University, New York, N.Y. 10021.

The avian sarcoma virus crk gene encodes a 47 kD fusion protein containing viral Gag sequences fused to cellular sequences containing SH2 and SH3 domains. Genetic and biochemical evidence suggests that v-Crk can induce transformation of chicken embryo fibroblasts by influencing the activity of cellular proteins involved in growth regulation. In this report, we have developed an in vitro microtiter assay to study the binding of bacterially expressed GST-fusion proteins of v-Crk and its cellular homolog, c-Crk, to the phosphorylated epidermal growth factor receptor (EGFR). Competitive binding data is presented that compares the inhibitory capacities of heterologous GST-fusion proteins containing GAPSH2[N], ablSH2, srcSH2, and PLCSH2[N] sequences. Results indicate that both full-length Crk and GAPSH2[N] bind the phosphorylated EGFR with high affinity and can quantitatively compete the binding of each other in a competitive ELISA. Binding of full length Crk or the isolated SH2 domains of GAP or abl resulted in a significant protection against dephosphorylation by cellular phosphatase activity. To extend these findings to pp130, the major phosphotyrosine-containing protein in CT10-transformed cells, we utilized a nitrocellulose filter-binding assay. Results demonstrate high affinity binding of Crk towards denatured pp130, and as is the case for phosphorylated EGFR, Crk binding can partially protect pp130 from phosphatase activity. However, no apparent competition of Crk binding was noted with heterologous SH2-containing constructs including GAPSH2[N], suggesting a possible specificity of Crk-pp130 interaction. These data are consistent with a direct role of Crk in the modulation of cellular phosphotyrosine status in vivo.

H 304 CORRELATION OF STRUCTURE / FUNCTION WITH ONCOGENIC POTENTIAL OF *ras* PROTEINS, Joel Bolonick, Gideon Bollag and Frank McCormick, Department of Molecular Biology, Cetus Corporation, Emeryville, CA 94608

Mutations at codon 61 of *ras* genes result in proteins with varying oncogenic potential. Previously, the transforming properties have been linked to reduced GTPase activity of the mutant proteins, and indeed they are insensitive to GTPase activating proteins. However, this simple interpretation cannot explain the wide variation in oncogenic potential.

In order to develop a molecular understanding of oncogenic potential, the biochemical properties of proteins with seven different amino acids at position 61 were compared. A clear correlation was noted between the nucleotide dissociation rates and oncogenic potency. Coupled with the reduced GTPase rates, the nucleotide dissociation results predict that the differing transforming properties depend on the steady state ratio of GTP:GDP bound to the mutant proteins.

The structural basis for the differences in nucleotide dissociation rates was investigated using molecular modeling techniques. Direct correlations were found between the predicted position of glycine 60 and the nucleotide dissociation rates. In particular, the dissociation rate of GTP correlated with the strength of the hydrogen bond between the amide hydrogen of glycine 60 and the γ-phosphate of GTP. On the other hand, the GDP dissociation rate correlated with repulsion between the carbonyl oxygen of glycine 60 and the β-phosphate of GDP. These results suggest that the amino acids at position 61 mediate their effects on nucleotide dissociation via the conformation of glycine 60.

This study concludes that the oncogenic potency of these *ras* mutants is determined by the steady state flux of nucleotides, and not simply by a block in GTPase activity as previously proposed. This conclusion has important implications for the *in vivo* regulation of *ras* proteins.

H 305 TYROSINE PHOSPHORYLATION OF THE T CELL RECEPTOR (TCR) ζ SUBUNIT: EVIDENCE FOR A MODULATING GTP BINDING PROTEIN. Cristina Cenciarelli, Robert Hohman, Fabian Gusovsky, and Allan M. Weissman. Experimental Immunology Branch, NCI, NIH Bethesda, MD 20892

The TCR is a complex multisubunit structure that recognizes foreign antigens via clonally derived heterodimeric recognition elements. These heterodimers exist in association with a set of invariant subunits. Receptor cross-linking leads to an increase in phosphoinositide turnover and subsequent protein kinase C (PKC) activation. In addition, receptor occupancy leads to the independent activation of one or more non-receptor tyrosine kinases. Among the most prominant tyrosine kinase substrates is the ζ subunit of the T cell antigen receptor. Upon receptor occupancy a subset of ζ molecules undergoes multiple cooperative phosphorylations. To dissect the molecular mechanisms that regulate and couple to ζ phosphorylation we examined tyrosine phosphorylation of ζ in a permeabilized cell system. Murine T cell hybridoma cells were permeabilized with staphylococcal α-toxin in a potassium glutamate based buffer system. Upon TCR cross-linking ζ phosphorylation occurred. The addition of the nonhydrolyzable GTP analogue GTPγS resulted in a substantial increase in the level of phospho-ζ. Similar increases were seen with GppNHp.The increases seen with both of these reagents were dose dependent and comparable in magnitude. In contrast, GDPβs did not increase, and often decreased, the level of ζ phosphorylation. Two nonhydrolyzable ATP analogues failed to induce a similar increase in cellular phospho-ζ. We assessed whether PKC might be playing a role in this increase in ζ phosphorylation. The GTPγS effect was not mimicked by phorbol esters nor was it significantly effected by overnight depletion of PKC prior to permeabilization. Together these results suggest that GTP binding proteins may play a role in the coupling to, or regulation of, tyrosine phosphorylation of this key T cell receptor component. Further studies are underway to define the molecular mechanisms responsible for these observations.

H 306 RAPID ACTIVATION OF A CYTOSOLIC TYROSINE PROTEIN KINASE IN RESPONSE TO EPIDERMAL GROWTH FACTOR. Claude Cochet*, Odile Filhol*, G.N. Gill° & Edmond M. Chambaz*, *INSERM U244, DBMS/BRCE, CENG,85X, 38041 Grenoble Cedex, France.°Dept of Medicine, Division of Endocrinology & Metabolism, Univ. of California, San Diego, La Jolla, 92093 USA.

We used tyrosine containing peptides to identify protein kinases activated in EGF-treated cells expressing different EGF receptor mutants.
Here, we present the characterization of a cytosolic tyrosine kinase which is rapidly activated by EGF. A massive and stable activation of the kinase was also achieved after the treatment of cells with orthovanadate. The kinase seems to be activated by phosphorylation on tyrosine since it is retained on an antiphosphotyrosine antibody column and almost completly deactivated upon incubation with a tyrosine specific protein phosphatase. Gel filtration analysis indicated that the kinase eluted as a large protein with an apparent molecule-weight of 350 kDa. However, the enzyme was found to coeluate on different chromatographic columns with a Mr 48 kDa protein phosphorylated on tyrosine residues. We found that among different substrates, the enzyme was able to phosphorylate a synthetic peptide corresponding to the regulatory phosphorylation sites in MAP kinase. However, deactivated MAP kinase could not be phosphorylated and reactivated by the enzyme. It is conceivable that this cytosolic tyrosine protein kinase may represent a link between the EGF receptor and serine/threonine protein kinases which act downstream in the mitogenic signal transduction cascade. Further progress toward the purification of the enzyme will be required to establish its function during signal transduction by growth factor receptors.

H 307 α-Thrombin stimulates tyrosine phosphorylation of GAP-associated proteins in IIC9 hamster fibroblasts. Simon J. Cook & Frank McCormick. Department of Molecular Biology, Cetus Corporation, 1400, 53rd Street, Emeryville, CA 94608, USA.

α-Thrombin is a potent mitogen that couples to at least two signal transduction pathways; (A) the hydrolysis of polyphosphoinositides to generate Ins(1,4,5)P_3 and sn-1,2-DG and (B) the inhibition of adenylate cyclase. Accordingly, the α-thrombin receptor has been cloned and identified as belonging to the class of G-protein -coupled receptors possessing seven transmembrane domains. Recent studies suggest that these two pathways alone cannot account for the mitogenic effects of α-thrombin.

The p21*ras* GTPase activating protein (p120-GAP) is phosphorylated upon tyrosine residues in response to stimulation with PDGF, EGF and CSF-1 and associates with two other major tyrosine phosphoproteins, p190 and p62. p120-GAP interacts with receptor tyrosine kinase signal transduction pathways; to date there is less evidence for a role for *ras* or p120-GAP in the signal transduction pathways generated by G-protein -coupled receptors. We show that α-thrombin stimulates the tyrosine phosphorylation of p120-GAP and p190, though more weakly than PDGF. In addition a 90 kDa protein present in anti-GAP immunoprecipitates is strongly phosphorylated upon tyrosine residues in response to α-thrombin, but less so by PDGF and EGF. Addition of phorbol-12-myristate-13-acetate (PMA) to quiescent IIC9 cells resulted in a similar pattern of tyrosine phosphorylation as that obtained with α-thrombin.

These results suggest that in addition to receptor tyrosine kinases, mitogens which activate G-protein coupled receptors may be able to interact with the *ras* pathway of cell proliferation.

H 308 GROWTH CONTROL BY OVER- AND UNDEREXPRESSION OF THE PROTO-ONCOGENE/PROTEIN SYNTHESIS INITIATION FACTOR eIF-4E. Arrigo De Benedetti, Carrie W. Rinker-Schaeffer,and Robert E. Rhoads, Department of Biochemistry, University of Kentucky, Lexington, KY 40536.
The rate of cell growth and division is closely linked to the overall rate of protein synthesis. Control of the translation rate is exerted primarily at the level of initiation, and particularly at the step of mRNA binding to the 40S ribosomal subunit (RSU). The eIF-4 group of initiation factors is responsible for the melting of secondary structure at the 5'-UTR of mRNA and its transfer to the 40S RSU. The the least abundant of these, eIF-4E, binds the mRNA cap and accompanies the transfer of mRNA to the 40S RSU. eIF-4E is a phosphoprotein whose phosphorylation correlates with cell growth rate. Stimulation of quiescent cells by a variety of mitogens leads to an increase in eIF-4E phosphorylation.

Studies from our laboratory have shown that overexpression of eIF-4E in HeLa cells promotes the formation of rapidly growing foci and causes loss of contact inhibition. Overexpression of a non-phosphorylatable variant of eIF-4E, however, is phenotypically null. Furthermore, continuous rat embryo fibroblasts transformed with the T24 *ras* oncogene have a greatly increased level of eIF-4E phosphorylation. This suggests that the eIF-4E kinase (as yet unidentified) operates at a stage downstream from *ras* activation. Decreasing the level of eIF-4E by production of antisense RNA results in slowed growth, reduction in protein synthesis, and coordinate loss of the p220 component of eIF-4F. However, the translation of some mRNAs, particularly those encoding heat shock proteins, are not affected by the loss of eIF-4E and p220. This suggests that some cellular mRNAs bind to the 40S RSU via a cap-independent pathway. The possibility of manipulating the levels of eIF-4E *in vivo* offers a unique opportunity to control events that are downstream in the mitogenic activation pathway and may allow intervention in the cell proliferative responses that are beyond the step of expression of an oncogene. (Supported by grants GM20818 from the NIGMS and 3076 from the Council for Tobacco Research - U.S.A., Inc.)

H 309 PHORBOL ESTERS DIFFERENTIALLY EFFECT PKC-α AND PKC-ε IN HUMAN LUNG CARCINOMA (A549) CELLS, Nicholas M. Dean, *Doriano Fabbro and C. Frank Bennett, ISIS Pharmaceuticals, 2280 Faraday Ave., Carlsbad, CA 92008, USA and *Research Dept. Pharmaceuticals Division, CIBA-GEIGY Limited, Basle, Switzerland.
Protein kinase C (PKC) consists of a family of protein serine/threonine kinases which can be sub-divided into two major groups, those with Ca^{2+} binding domains (C2), (PKC-α,βI,βII and γ) and those without (PKC-δ,ε,ζ,η), suggesting a different requirement of each for calcium during activation. We have examined the localization and regulation by phorbol esters of PKC-α and PKC-ε as examples from each group and have determined that major differences between the two exist. In A549 cells PKC-α protein measured by Western blotting is largely, (80-90%), cytosolic, and rapidly translocates to the membrane upon addition of phorbol 12,13-dibutyrate (PDBu). After 12 hours of PDBu treatment PKC-α protein levels are reduced to between 10-20% of control. Northern blotting of total A549 RNA with a PKC-α specific probe revealed two major transcripts of 8.5kb and4.0kb, both of which are increased two fold by PDBu treatment. In contrast to PKC-α, PKC-ε protein is predominantly membrane associated, and both levels and localization are uneffected by short term (≤12 hours) incubation of cells with PDBu. Long term (48 hours) incubation of cells results in a gradual decrease in PKC-ε protein levels of about 50%. These results demonstrate that PDBu-induced proteolytic degradation of isozymes of PKC proceeds at different rates and suggest that levels of individual isozymes can be differently regulated.

H 310 RECEPTOR STIMULATION OF NUCLEAR DIACYLGLYCEROL (DAG) FORMATION. Nullin Divecha, Hrvoje Banfic and Robin F. Irvine, Department of Biochemistry, AFRC Institute of Animal Physiology and Genetics Research, Babraham Hall, Cambridge, CB2 4AT, U.K.

Rapid labelling studies using γ^{32} P ATP and isolated 3T3 nuclei have previously suggested changes in the metabolism of nuclear inositol lipids(Cocco *et al.* (1987) Biochem. J. 248 765-770). To investigate this further we have developed picomole-sensitive mass assays for PtdIns, Ptd (4)*P* , PtdIns (4,5)P_2 and DAG, and have characterized the mass changes in nuclear lipids on stimulation of 3T3 cells with insulin-like growth factor-1 (IGF-1).

We have demonstrated a rapid breakdown of PtdIns (4,5)P_2 (within 2 min.) with a concomitant rise in nuclear DAG.The mass levels of these lipids returned to control levels within 1 hour. Immunoblotting studies with an antibody to protein kinase C (PKC) demonstrated a rapid translocation of this enzyme to the nuclear fraction following the same time-course as the elevation of nuclear DAG. Parallel experiments with bombesin, which stimulates plasma membrane located PI C, showed no changes in PtdIns (4,5)P_2 , DAG or the translocation of PKC to the nucleus.

We take this data to suggest that IGF-1 in 3T3 cells causes a rapid stimulation of nuclear phosphoinositidase C (PIC) resulting in an increase in nuclear DAG, leading to the translocation and possible activation of PKC within the nucleus.

H 311 REGULATION OF THE ACTIVITY OF p21^{ras} IN T-LYMPHOCYTES. J.Downward, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX.

We have demonstrated 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 control of the T-cell antigen receptor and also the interleukin 2 receptor. Protein kinase C mediates at least part of this stimulation of p21^{ras} which appears to occur through the suppression of "GAP"-like 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 groups of proteins, GAP/NF-1 and guanine nucleotide exchange factors. The relative contribution of GAP and of NF-1 to the regulation of p21^{ras} in T-cells is being investigated. The role of phosphorylation of NF-1 and GAP, their association with other proteins and with lipids on the regulation of p21^{ras} will be discussed.

H 312 ANALYSIS OF RAS GAP FUNCTION THROUGH RETROVIRAL MEDIATED DELIVERY OF WILD TYPE AND MUTANT GAP GENES INTO RAT 1 CELLS. Sean E. Egan and R. A. Weinberg. Whitehead Institute for Biomedical Research and Department of Biology, M.I.T., Cambridge, MA 02142

Ras genes encode 21kd protein products which in their active GTP-bound state are capable of stimulating cell division and transformation. Two proteins have been described which are capable of stimulating the GTPase of ras proteins and therefore deactivating them. These two proteins are GAP and NF1. In an order to study biological function(s) of the GAP gene in signal transduction we have attempted to overexpress both wild type and mutant GAP genes in rodent fibroblasts. Initial efforts were not very successful and therefore a new statagy has been devised for expression of growth suppressive genes. This technique has been used to express wild type human GAP in Rat 1 fibroblasts. Cell lines have been generated following infection with a GAP/puromycinR virus. Many of these lines are growth suppressed and resistant to transformation by tyrosine kinase oncogenes but not by the serine/threonine kinase v-raf. In addition, cell lines have been generated through infection with vectors which contain isolated domains of GAP such as the SH2 regions and the GTPase activating C-terminal domain. These cell lines show a similar growth impaired phenotype as do cells infected with an NF1 catalytic domain containing retrovirus. All of the above lines will be analyzed for their sensitivity to transformation by a series of matched oncogenic viruses containing the hygromycin resistance gene as well as one of raf, ras, src, or Neu oncogenes.

H 313 SIGNAL TRANSDUCTION BY THE MITOGENIC PHOSPHOLIPID LPA

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Lysophosphatidic acid (LPA; 1-acyl-glycerol-3-phosphate), a naturally occuring phospholipid that is present in plasma and serum, can evoke such diverse cellular processes as platelet aggregation, Dictyostelium chemotaxis, smooth muscle contraction and, in fibroblasts, cell proliferation. LPA initiates its action at the external surface of the plasmamembrane and stimulates the following signalling pathways: (1) GTP-dependent activation of phospholipase C; (2) breakdown of phosphatidylcholine via phospholipase D activation; (3) release of arachidonic acid; and (4) G_i mediated inhibition of adenylate cyclase. Furthermore, LPA induces distinct changes in membrane potential and conductance via an as yet unknown mechanism.

In neuronal cells, LPA also causes immediate changes in the actin cytoskeleton resulting in cell rounding, as if cells prepare for mitosis. LPA-induced cell rounding can not be attributed to the above-mentioned second messenger systems. Instead it is blocked by both pervanadate and microinjected vanadate, tyrosine phosphatase inhibitors. Rounding is accompanied by an increase in pp60$^{c\text{-}src}$ activity in a vanadate sensitive manner. The results suggest a model in which extrinsic LPA not only activates certain G proteins, but also stimulates (neuronal) pp60$^{c\text{-}src}$ activity via an unidentified phosphatase activity, which could be responsible for the premature mitotic phenotype.

H 314 CO-EXPRESSION OF BOTH α AND ß SUBUNITS IS REQUIRED FOR ASSEMBLY OF REGULATED CASEIN KINASE II. Odile Filhol*, Claude Cochet*, Phill. Wedegaertner°, G.N. Gill° & Edmond M. Chambaz*, *INSERM U244, DBMS/BRCE, CENG,85X, 38041 Grenoble Cedex, France. °Dept of Medicine, Division of Endocrinology & Metabolism, Univ. of California, San Diego, La Jolla, 92093 USA. Casein kinase II (CKII) is an ubiquitous serine-threonine kinase whose functional significance and regulation in the living cell are not clearly understood. The native enzyme has an oligomeric structure made of two different (a and ß) subunits with an $\alpha_2\beta_2$ stoichiometry. To facilitate the study of the structure-activity relationship of the kinase, we have expressed its isolated subunits in a baculovirus-directed-insect cell expression system. The resulting isolated recombinant α subunit exhibited a protein kinase catalytic activity, in agreement with previous observations (*Cochet & Chambaz, J. Biol. Chem., 1983, 258, 1403-1406*). Co-infection of insect cells with recombinant viruses encoding the two kinase subunits resulted in the biosynthesis of a functional enzyme. Active recombinant oligomeric kinase was purified to near homogeneity with a yield of about 5 mg of enzymatic protein per liter, showing that, in co-infected host cells, synthesis was followed, at least in part, by recombination of the two subunits with an $\alpha_2\beta_2$ stoichiometry. The catalytic properties of the recombinant enzyme appeared highly similar to those previously observed for casein kinase II purified from bovine tissue. Access to the isolated subunits and to their $\alpha_2\beta_2$ association disclosed that the ß subunit is required for optimal catalytic activity of the kinase. In addition, the ß subunit is suggested to play an essential role in the regulated activity of the native casein kinase II. This is clearly illustrated by the observation of the effect of spermine which requires the presence of the ß subunit to stimulate the kinase catalytic activity which is born by the a subunit. Production of the oligomeric enzyme by co-infected insect cells should provide a powerful experimental system to study structural modifications that may be responsible for the regulation of casein kinase II functions, in living cell.

H 315 UNOHOSPHORYLATED α-PKC EXHIBITS PHORBOL ESTER BINDING BUT LACHS PROTEIN KINASE ACTIVITY Filipuzzi, I., Fabbro, D.* and Imber, R., Molecular Tumorbiology, Department of Research, University School of Medicine, CH-4031 Basel, Switzerland, *CIBA GEIGY Pharmaceutical Research Division K 125 405, 4002 Basel, Switzerland.
We have previously shown that the α-isoform of protein kinase C (α-PKC) is synthesized as a precursor molecule which is then phosphorylated yielding the mature enzyme form. Here, we demonstrate that the expression of α-PKC in E.coli led to the accumulation of the unphosphorylated form. This precursor was purified and found to exhibit phorbolester binding but no protein kinase activity. In addition, the precursor molecule was able to specifically interact with phosphatidyl serine, threonine and the ATP analogon Ciba-cron Blue F3G-A, respectively, indicating that phosphorylation of α-PKC is neither required for binding of the substrate-ATP complex nor of the effector phosphatidyl serine or of phorbolester activators. Therefore, our results suggest that post-translational phosphorylation of α-PKC is only required for the transfer of phosphate residues to protein substrates, but not for binding of effector or substrate molecules.

H 316 HaRAS MEDIATES BOTH RAF-1-DEPENDENT AND RAF-1-INDEPENDENT INTRACELLULR SIGNALING PATHWAYS INITIATED BY V-SRC
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v-Src activates promoters under the control of serum response elements (SREs) and 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements (TREs) via two distinct intracellular signaling pathways. v-Src-induced SRE-dependent gene expression was via a protein kinase C (PKC)-independent/Raf-1-dependent pathway; whereas, v-Src-induced TRE-dependent gene expression was via a PKC-dependent/Raf-1-independent pathway. A dominant inhibitory mutant of HaRas blocked both SRE- and TRE-mediated gene expression induced by v-Src. Consistent with these results, the induction of SRE-dependent gene expression by an activated form of HaRas was indpendent of PKC and dependent upon Raf-1; and the induction of TRE-dependent gene expression by activated HaRas was dependent upon PKC and independent of Raf-1. These data suggest HaRas is an early signaling component upstream from either PKC or Raf-1 in multiple signals initiated by v-Src.

H 317 PHOSPHORYLATION AND ACTIVATION OF MAP2-K IN B LYMPHOCYTES FOLLOWING STIMULATION WITH CALCIUM IONOPHORES, Richard A. Franklin, Bruce D. Mazer, Joseph J. Lucas, and Erwin W. Gelfand, Dept. of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80210
Immunoglobulin receptor ligation results in the immediate breakdown of phosphatidylinositol bisphosphate leading to an increase in intracellular calcium and activation of protein kinase C as well as later events such as cell proliferation and immunoglobulin secretion. Tyrosine phosphorylation may play a critical role in linking the breakdown of phosphatidylinositol bisphosphate to these later events. We examined the involvement increases in intracellular calcium have in tyrosine phosphorylation. We report that stimulation with the calcium ionophore, ionomycin, induces the tyrosine phosphorylation of a protein, approximately 42 kDa in size, as detected by immunoblot analysis, in both a B lymphoblastoid cell line (LA350) and B lymphocytes purified from peripheral blood. Treatment of B lymphocytes with another calcium ionophore, A23187, also induced tyrosine phosphorylation of this protein, demonstrating that this response was not specific to ionomycin. Phosphorylation of this protein was not detectable within the adherent cells isolated from the peripheral blood, demonstrating that this response was not due to contaminating monocytes in our B lymphocyte preparations. Phosphorylation of this protein in B lymphocytes was rapid, detectable within 30 seconds, and transient, returning to background levels by 45 minutes. Treatment of LA350 or purified B lymphocytes with PMA, a known activator of microtubule associated protein 2-kinase (MAP2-K) in B lymphocytes, induced the tyrosine phosphorylation of a protein with the identical mobility as the protein phosphorylated following ionomycin stimulation. Lysates from ionomycin-stimulated purified B lymphocytes or LA350 cells exhibited greater than twice the ability to phosphorylate myelin basic protein, a substrate for activated MAP2-K, when compared to untreated cells. The activation of S6K-II following ionomycin stimulation is currently under investigation. Based on the protein's molecular weight, it's ability to be phosphorylated following PMA stimulation and the ability of lysates from ionomycin stimulated cells to phosphorylate myelin basic protein we suggest that this tyrosine phosphorylated protein is MAP-2K.

H 318 INVOLVEMENT OF TYROSINE PHOSPHORYLATION AND A G-PROTEIN IN THE ACTIVATION OF PHOSPHATIDYLCHOLINE SPECIFIC PHOSPHOLIPASE C BY HUMAN COLONY STIMULATING FACTOR 1 RECEPTOR

Goutam Ghosh Choudhury, Victor Sylvia and Alan Y. Sakaguchi, Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Intracellular signalling leading to normal cell growth and transformation by receptor tyrosine kinases (RTK) are incompletely understood. Activated RTKs stimulate hydrolysis of membrane phospholipids to generate intracellular second messengers that may be important for mitogenesis. RTKs such as PDGFR and EGFR phosphorylate a phosphoinositide (PI) specific phospholipase C at tyrosine residues and this tyrosine phosphorylation has been implicated in its enzymatic activity. In contrast, colony stimulating factor 1 receptor, a member of the PDGFR family of RTKs, does not stimulate PI turnover, but rather utilizes phosphatidylcholine catabolic pathway for producing second messengers [Ghosh Choudhury et. al. (1991) FEBS Letters, 282, 351-354]. Now we provide evidence that CSF1 stimulates a phosphatidylcholine specific phospholipase C (PC-PLC) in an antiphosphotyrosine affinity isolated protein fraction. This affinity isolated enzymatic activity is sensitive to tyrosine specific T-cell phosphatase suggesting that tyrosine phosphorylation is necessary for its function. Cell fractionation studies revealed that the CSF1 stimulated PC-PLC activity was present in the membrane. Antiphosphotyrosine affinity isolated PC-PLC activity was reduced in pertussis toxin pretreated cells. In isolated membranes the PC-PLC activity was sensitive to *in vitro* pertussis toxin treatment but stimulated by GTPγS. These results suggest involvement of a G-protein and tyrosine phosphorylation in the CSF1 mediated activation of PC-PLC.

H 319 SYSTEMATIC MUTATIONAL ANALYSIS OF THE cAMP-DEPENDENT PROTEIN KINASE IDENTIFIES REGIONS OF THE CATALYTIC SUBUNIT IMPORTANT FOR THE RECOGNITION OF THE REGULATORY SUBUNIT.

Craig S. Gibbs and Mark J. Zoller, Department of Protein Engineering, Genentech Inc, 460 Pt San Bruno Blvd, South San Francisco, CA 94080.

The regulatory subunit (R) of the cAMP-dependent protein kinase is thought to inhibit the catalytic subunit (C) by binding in a manner analogous to a protein substrate. The regulatory subunit contains an autoinhibitor sequence that conforms to the consensus sequence recognized in protein substrates (RRXS/THy), presumably the autoinhibitor sequence occupies the active site and acts as a competitive inhibitor of the catalytic subunit. A battery of mutants of yeast C was screened for mutants that were defective in the recognition of the R subunit. The mutants fell into two classes depending on whether they were also defective in the recognition of a peptide substrate (kemptide) that conforms to the consensus recognition sequence. The residues mutated were mapped onto the three-dimensional structure of the murine C subunit bound to an inhibitor peptide. Residues important for the recognition of both the R subunit and the peptide substrate mapped to the peptide binding site, functionally defining the binding site for the autoinhibitor sequence. Residues important only for the recognition of the R subunit defined an additional binding region for the R subunit on the surface of the C subunit in a region distal to the peptide/autoinhibitor binding site. Interactions between R and C at this site may be responsible for the high affinity binding of the R subunit. A key feature of this region is Thr-241, a site of autophoshorylation that is conserved in many protein kinases. This residue was previously shown to be important for the recognition of the R subunit, the substitution of all three residues that ligand to the phosphate group also affected R subunit binding. This autophosphorylation site has also been shown to be important for the activity of fps and src, and for the activation of the insulin receptor and cdc2. It is possible that this region is utilized by a variety of protein kinases for interactions with regulatory molecules or domains.

H 320 IDENTIFICATION AND CLONING OF A MAMMALIAN GENE WITH HOMOLGY TO YEAST GENES WHICH ACTIVATE RAS.

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The Ras proteins are membrane-bound proteins which bind and hydrolyze GTP. Mutations which either inhibit hydrolysis of GTP or facilitate guanine nucleotide exchange lead to oncogenic activation of the Ras gene product and are frequently found in tumors, suggesting that GTP-bound Ras proteins are involved in signal transduction. Proteins have been identified which control the cycling of Ras between the GTP and GDP-bound forms. GAP and NF1 in mammals and IRA1 and IRA2 in *S. cerevisiae* catalyze the hydrolysis of Ras-bound GTP, while CDC25 from *S. cerevisiae* and STE6 from *S. pombe* catalyze the the exchange of GDP for GTP. These proteins represent potential sites for transmitting signals to Ras in the control of cell growth and differentiation. By isolating a mammalian homolog of the yeast exchange factors, we hope to investigate the role of the exchange protein in normal and neoplastic growth.

We have cloned a portion of a mammalian gene which is homologous to STE6/CDC25 using the polymerase chain reaction (PCR) with degenerate primers corresponding to protein sequences conserved between STE6 and CDC25. The predicted amino acid sequence of the PCR-product is more closely related to those of the yeast Ras exchange proteins (25 to 29% identity) than to yeast exchange proteins which interact with other small G-proteins (11 to 18% identity), suggesting that the corresponding gene product could play a role in the regulation of mammalian Ras proteins. The transcript corresponding to this cDNA is found in all tissues that we checked (brain, liver, spleen, lymphocytes, NIH 3T3). To determine if this protein regulates Ras *in vivo*, we have cloned and sequenced the full-length cDNA and are using the cloned gene to test the *in vitro* and *in vivo* effect of the protein on Ras and other small G-proteins.

H 321 EFFECT OF THE ETHER LIPID ANALOGUE ET-18-OCH_3 ON PHOSPHATIDYLCHOLINE HYDROLYSIS IN NIH 3T3 AND HA-*RAS* TRANSFORMED NIH 3T3 CELLS,

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The ether lipid ET-18-OCH_3 is the prototype of a new class of non-DNA interactive anticancer drugs. ET-18-OCH_3 is a potent inhibitor of growth factor-induced inositol phosphate production and Ca^{2+} signalling (Seewald et al., 1990, Cancer Res.) and appears to inhibit specifically the PIP_2-PLC (Powis et al., submitted). We have now studied ET-18-OCH_3 for a potential activity against phospholipase D (PLD) and phosphatidylcholine phospholipase C (PC-PLC), because they may be involved in growth factor intracellular signalling. These phosphodiesterases act on phosphatidylcholine to generate choline and phosphatidic acid, and phosphocholine and diacylglycerol, respectively. In the present study, the release of choline and phosphocholine were examined in ^3H choline labelled NIH 3T3 cells as well as labelled membranes prepared from these cells and Ha-Ras transformed NIH 3T3. After 1 h incubation at 37°C NIH 3T3 cell membranes released 51.7 ± 3.8% phosphocholine and 32.9 ± 3.8% choline whereas Ha-*Ras* cell membranes released 22.9 ± 6.25% and 62.6 ± 6.3% of phosphocholine and choline, respectively. ET-18-OCH_3 inhibited this release with an IC_{50} of > 100 μM for NIH 3T3 and a IC_{50} = 80 μM for Ha-Ras NIH 3T3. In whole cells short-term treatment (30 min) with ET-18-OCH_3 up to 20 μM did not affect the level of extracellular or intracellular choline and phosphocholine. ET-18-OCH_3 above 20 μM caused a small increase in total phosphocholine formation and at the same time a redistribution of the phosphocholine and choline from inside to outside the cell was observed, presumably due to an increase in cell membrane permeability. From these studies, we conclude that ET-18-OCH_3 which inhibits cell growth of NIH 3T3 (IC_{50} = 20 μM) and of Ha-Ras cells (IC_{50} = 40 μM) has little effect on the PLD and PC-PLC activity. Although the specific inhibition of the PIP_2-PLC could be involved in the cytotoxicity of this drug, others mechanisms of action of the ET-18-OCH_3, and derivatives, cannot be ruled out and remain to be determined. Supported by NCI Grant CA 42286.

H 322 REQUIREMENTS FOR ACTIVATION OF THE C-*YES* PROTO-ONCOGENE Heidi Greulich, Melanie C. O'Brien, Hidesaburo Hanafusa, and Marius Sudol; The Rockefeller University, New York, NY 10021

The v-*yes* oncogene was isolated from the Y73 chicken sarcoma virus, a replication defective retrovirus. The cellular counterpart of the viral oncogene, c-*yes*, is a member of the *src* protein tyrosine kinase family, displaying 70% overall homology with *src*. Unlike the c-Yes protein, v-Yes is expressed as a gag fusion. Fusion occurs at the first codon of the Yes protein, and the initial methionine is changed to valine. In addition, v-Yes contains two internal point mutations and has an altered carboxyterminal tail. The altered tail sequence removes tyrosine 535, the putative regulatory tyrosine analogous to Src tyrosine 527.

We first investigated whether overexpression of c-Yes, with or without gag fusion, could transform chicken embryo fibroblasts. We made gagless c-Yes by replacing *src* with c-*yes* in Rous sarcoma virus, and made a gag-c-Yes fusion protein by replacing the v-*yes* gene in Y73 with c-*yes*. Neither of these overexpressed proteins transformed chicken embryo fibroblasts, as shown by lack of morphological alteration or growth in soft agar. However, the specific kinase activity of overexpressed c-Yes was no higher than that of endogenous c-Yes.

In order to test whether elevated kinase activity is required for c-Yes activation, we replaced c-Yes tyrosine 535 with phenylalanine in the Y73 gag fusion vector system. The specific kinase activity of the mutated protein is elevated, and the construct is transforming in chicken embryo fibroblasts. A c-Yes / v-Yes chimera yielded the same result.

H 323 THE DROSOPHILA *Roughened* MUTATION; ACTIVATION OF A *rap* HOMOLOG DISRUPTS CELL DETERMINATION IN THE DEVELOPING EYE. Iswar K. Hariharan, Richard W, Carthew and Gerald M. Rubin. Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720.

The *Roughened* mutation displays dominant inheritance and results in flies having rough eyes. In most of the ommatidia of *Roughened* flies, there are missing photoreceptor cells. Most commonly, the R7 photoreceptor cell is missing. These abnormalities appear to result from misspecifications in cell fate early in the development of the eye.

The *Roughened* mutation is characterized by a *Phe to Leu substitution at position 157 of the Rap 1* gene. The Drosophila *Rap1* gene has 88% amino acid identity with the human *rap1a* gene. The *Roughened* mutation appears to activate the *Rap1* gene. Loss of function *Rap1* alleles are lethal.

In mammalian cells, the *ras* and *rap* genes appear to have antagonistic functions. This interaction may mirror that observed in the Drosophila eye. The *Ras1* gene is part of the pathway involved in R7 determination. Activation of *Rap1* may interfere with R7 determination by antagonizing this pathway.

It will now be possible to identify genes whose products interact with the *Rap1* polypeptide using a simple genetic screen. Mutations that are dominant enhancers or suppressors of the *Roughened* phenotype will be sought and characterized.

H 324 **EXPRESSION AND TYROSINE PHOSPHORYLATION OF p42 MAP KINASE MUTANTS**, Jeng-Horng Her, Gilles L'Allemain, Jie Wu*, Anthony J. Rossomando, Robert L. Del Vecchio, Thomas W. Sturgill* and Michael J. Weber, Department of Microbiology and Internal Medicine and Pharmacology*, Health Sciences Center, University of Virginia, VA 22908

Mitogen-activated protein kinase (MAP kinase) is a ser/thr kinase that is activated by tyrosine and threonine phosphorylation following treatment of quiescent cells with a variety of stimulatory agonists, and its regulatory phosphorylation is likely to be important in signaling pathways involved in growth control, secretion and differentiation. To study the mechanisms by which MAP kinase becomes phosphorylated and activated, we have cloned a full-length cDNA encoding MAP kinase and have expressed the enzyme in E. coli and COS cells. The enzyme expressed in bacteria as a soluble nonfusion protein shows an apparent molecular weight of 42 kDa and a basal, intramolecular autophosphorylation on tyrosine 185 that is accompanied by partial activation of the enzyme's kinase activity towards an exogenous substrate. The protein expressed in COS cells displays chromatographic properties identical to the endogenous p42 MAP kinase. In addition, it becomes tyrosine-phosphorylated and enzymatically active. We have also constructed a kinase-defective MAP kinase mutant and mutants with the regulatory phosphorylation sites being replaced with nonphosphorylatable amino acids and provided evidence for an agonist-stimulated activity which enhances the tyrosine autokinase activity of p42 MAP kinase as well as for a kinase which phosphosrylates tyrosine 185 independent of p42 MAP kinase autokinase activity. Thus, regula-tory tyrosine phosphorylation of p42 MAP kinase occurs by at least two cellular mechanisms.

H 325 ACTIVATION OF PLCγ1 CATALYTIC ACTIVITY BY PHOSPHATIDIC ACID, Gwenith Jones and Graham Carpenter, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN. 37232.

We investigated the possibility that lipids may have a modulatory influence on PLCγ1 activity. PLCγ1 activity was found to be stimulated by anionic phospholipids. Phosphatidic acid (PA) had the greatest stimulatory effect, followed in order by lysophosphatidic acid >> phosphatidylglycerol > phosphatidyl serine. Diacylglycerol had no effect on activity for either enzyme. PA stimulated both the activity of the unphosphorylated and phosphorylated form of the enzyme, though the magnitude of stimulation was greater for unphosphorylated (60-fold) than the phosphorylated (24-fold). Using a mixed micelle assay system, the effect of PA on PLCγ1 kinetic parameters was determined. PA had no effect on V_{max}, but did lower the K_m for PIP_2 for both the unphosphorylated and phosphorylated form of the enzyme. In the absence of PA, the $S_{0.5}$ was determined to be 0.8 and 0.4 mole fraction for unphosphorylated and phosphorylated PLCγ1, respectively. In the presence of PA, the K_m was 0.1 and 0.02 mole fraction, respectively. PA lowered Ks for the micelle 2-fold for both forms of the enzyme. A physiological role for PA in intact cells was investigated. EGF treatment resulted in a transient increase in PA production, that peaked at two minutes. This data suggests that PA may play a role in the activation of membrane-associated form of PLCγ1. (This work was supported by NIH grants PO1 CA 43720 and T32 CA09582).

H 326 *DROSOPHILA src* BEHAVES LIKE AN ACTIVATED TYROSINE KINASE WHEN OVEREXPRESSED IN *DROSOPHILA* SCHNEIDER 2 CELLS, Steven J. Kussick* and Jonathan A. Cooper, Fred Hutchinson Cancer Research Center and *University of Washington School of Medicine, Dept. of Pathology, 1124 Columbia St., Seattle, WA 98104.

We have studied the relationship between the phosphorylation state of the *Drosophila src* 64B (D*src*) gene product, $p62^D$, and its tyrosine kinase activity, to determine if $p62^D$ is regulated like the vertebrate *src*-family kinases. To map $p62^D$ phosphorylation sites in *Drosophila* cells, wild-type and mutated D*src* constructs driven by the hsp70 promoter were overexpressed in Schneider 2 cells by transient transfection. Overexpressed wild-type $p62^D$ was detectably phosphorylated only at tyrosine. Two-dimensional tryptic and chymotryptic phosphopeptide mapping showed that the C-terminal tyrosine (Tyr 547) of $p62^D$ was phosphorylated *in vivo*, like the C-terminal tyrosine of vertebrate $p60^{c\text{-}src}$ in fibroblasts. However, unlike $p60^{c\text{-}src}$, wild-type $p62^D$ was also phosphorylated at additional tyrosines *in vivo*, and the stoichiometry of these phosphorylations was broadly similar to the stoichiometry of Tyr 547 phosphorylation. These additional tyrosines were autophosphorylation sites *in vitro*, and one of them is the $p62^D$ homolog of the $p60^{c\text{-}src}$ Tyr 416 autophosphorylation site. A catalytically-inactive $p62^D$ mutant was phosphorylated at the C-terminus but not at the autophosphorylation sites, suggesting that Tyr 547 phosphorylation occurs in *trans* in Schneider 2 cells.

Overexpression of wild-type $p62^D$, or a variety of catalytically-active $p62^D$ mutants, significantly increased the phosphorylation of numerous Schneider cell proteins on tyrosine, while expression of catalytically-inactive mutants of $p62^D$ had no such effect on cellular phosphotyrosine. Taken together, the peptide mapping and antiphosphotyrosine blot results suggest that, in contrast to the repression of $p60^{c\text{-}src}$ activity in fibroblasts, $p62^D$ is catalytically active when overexpressed in *Drosophila* cells, perhaps because of sub-stoichiometric C-terminal tyrosine phosphorylation.

H 327 THROMBIN INDUCED ASSOCIATION OF A PHOSPHOLIPASE $C\gamma$-GTPase ACTIVATING PROTEIN COMPLEX WITH RAP1b IN HUMAN PLATELETS. Eduardo G. Lapetina and Mauro Torti. Division of Cell Biology, Burroughs Wellcome Co., Reasearch Triangle Park, NC 27709

Phospholipase C catalyzes the hydrolysis of inositol phospholipids and leads to the formation of intracellular messengers that regulate protein kinase C and levels of cytoplasmic Ca^{++}. Four different phospholipases C, referred to as α, β, γ, and δ, have been identified in several mammalian tissues. Phospholipase $C\gamma$ plays a role in growth factor signal transduction and is able to physically associate with the activated PDGF receptor, PI3-kinase, and $p21^{ras}$-GTPase activating protein, GAP. Thrombin causes rapid activation of phospholipase C in human platelets, concommitent with aggregation and secretion. Using specific polyclonal antiserum and monoclonal antibodies we found that human platelets express both phospholipase $C\gamma$ and rasGAP. A significant amount of phospholipase $C\gamma$ was detected in the rasGAP immunoprecipitates from both resting and thrombin-stimulated platelets. Using ^{32}P labeled platelets, it was evident that the phospholipase $C\gamma$ bound to rasGAP is phosphorylated. Platelets possess insignificant amounts of $p21^{ras}$ and therefore the role of rasGAP in these cells is unclear.RapGAP, however can bind with high affinity to the ras-related protein rap1b, without stimulating it's intrinsic GTPase activity.We have found that in thrombin-stimulated platelets rap1b forms a complex with rasGAP and phospholipase $C\gamma$. These results demonstrate that phospholipase $C\gamma$ and rasGAP are physically associated in quiesent platelets and that stimulation with thrombin induces this complex to associate with activated rap1b (GTPrap1b). It is therefore possible that activation of platelet phospholipase $C\gamma$ by thrombin occurs through interaction with rasGAP. Initially, thrombin causes the binding of GTP to rap1b, which associates with the phospholipase $C\gamma$ - rasGAP complex. This probably allows access of phospholipase $C\gamma$ to membrane-bound inositol phospholipids.

H 328 cAMP DEPENDENT STIMULATION OF TYPE II PHOSPHOLIPASE A_2 BY IL-1β AND TNFα IN RAT SYNOVIAL CELLS

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IL-1β and TNFα have pleiotropic and partially overlapping effects on both normal and tumoral cells but their mechanism of action is not completely understood.In primary cultures of fibroblast-like rat synovial cells IL-1β and TNFα stimulate type II phospholipase A_2 (PLA_2) activity in a dose and time dependent manner.Second messenger pathways involved in this action were investigated. First, agents known to increase the intracellular concentration of cyclic AMP were used. Dibutyryl cyclic AMP, but not dibutyryl cyclic GMP, caused a dose dependent increase of PLA_2 activity in the media. Forskolin showed the same effect but its analogue 1,9-dideoxyforskolin, inactive on adenylate cyclase, was ineffective. Furthermore, forskolin potentiated the effects on PLA_2 of IL-1β and TNFα. Cholera toxin increased PLA_2 activity in a dose dependent manner. Pertussis toxin showed a small effect alone but shifted to the left the dose response curves to forskolin, IL-1 and forskolin plus IL-1. The phosphodiesterase inhibitors IBMX, rolipram, zaprinast and CI930 were ineffective when added alone but increased the effects of IL-1β and TNFα with a rank of potency (rolipram>IBMX>CI930>zaprinast) consistent with a cyclic AMP mediated process.Protein kinase (PK) inhibitors were used to show a direct involvment of PK's in the transduction pathway of IL-1 and TNF. KT5720, a relatively specific inhibitor of PK-A, inhibited IL-1β and TNFα effects on PLA_2 activity. KT5823, an inhibitor more specific for PK-G, did not influence the response of the synovial cells. Finally, indomethacin, a cycloxygenase inhibitor, did not affect the action of IL-1 and TNF, ruling out an action by endogenous PGE_2.

Thus, IL-1β and TNFα act through a cyclic AMP dependent pathway in the stimulation of the type II PLA_2 in rat synoviocytes.

H 329 LIPIDS INCLUDING SECOND MESSENGERS MODULATE AND PHYSICALLY INTERACT WITH N-CHIMAERIN, A $p21^{rac}$ GTPase ACTIVATING PROTEIN, Joel Lee[#], Sohail Ahmed[#], Robert Kozma[#], Mabel Teo[#], Clinton Monfries[#] and Louis Lim[#].
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Human brain specific n-chimaerin cDNA[1] encodes a phospholipid-dependent phorbol ester/diacylglycerol receptor[2] at the N terminus and a $p21^{rac}$ GTPase activating protein (GAP)[3] at the C terminus. The cysteine-rich domain of n-chimaerin with the $HX_{12}CX_2CX_{13}CX_2CX_4HX_2CX_7C$ motif present at the N terminus has sequence identity to the C1 domain of protein kinase C. Zinc binds to this region and is required for phorbol ester binding[4]. The C terminus of n-chimaerin has identity to regions of $p21^{rac}$ GAP and the product of the bcr (breakpoint cluster region) gene; all 3 proteins have $p21^{rac}$ GAP activity. The biochemical property afforded by the N terminus suggest the possibility that lipids including second messengers might regulate the GAP activity of n-chimaerin.

We will present data on the effects of lipids including second messengers on the GAP activity of n-chimaerin proteins purified from Escherichia coli. Our findings suggest that lipids including second messengers physically interact and modulate the GAP activity of n-chimaerin through the N terminus.

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H 330 EVIDENCE THAT EXTRACELLULAR SIGNAL-REGULATED KINASES (ERKS) ARE THE INSULIN-ACTIVATED Raf-1 KINASE KINASES, Ruey-min Lee*, Melanie H. Cobb#, and Perry J. Blackshear*, the *Howard Hughes Medical Institute Laboratories, Duke University Medical Center, Durham, NC 27710 and the #Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9041

The Raf-1 proto-oncogene protein kinase can be phosphorylated and activated after stimulation of cells with insulin and a variety of other growth factors and mitogens. We identified four peaks of Raf-1 kinase kinase activity by anion exchange chromatography of cell lysates; two of these were activated by insulin. Further chromatographic separation of these two peaks of insulin-activated kinase activity indicated that they contained three apparently distinct kinase activities. Two of these activities comigrated with immunoreactive extracellular signal-regulated kinases (ERK) 1 and 2 (MAP kinase) through three different chromatographic separations. Both ERK1 and ERK2 phosphorylated Raf-1 with reasonably high affinity (K_m for ERK1= 90nM; K_m for ERK2= 120nM), and produced similar, complex phosphopeptide maps. The third kinase activity also phosphorylated Raf-1 and myelin basic protein, but did not comigrate exactly with either immunoreactive ERK1 or ERK2. We conclude that two and possibly three insulin-activated Raf-1 kinase kinases are members of the ERK family. Identification of the phosphorylation sites is currently underway.

H 331 SH2-MEDIATED INTRA- AND INTER-MOLECULAR INTERACTIONS OF THE SRC TYROSINE KINASE. Xingquan Liu, Scott R. Brodeur[1], Andrew P. Laudano[1], C. Anne Koch and Tony Pawson. Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Canada M5G 1X5. 1. Department of Biochemistry, University of New Hampshire, Durham, New Hampshire 03825, USA

The enzymatic and transforming activities of the proto-oncogene product, $p60^{c\text{-}src}$, can be activated either by removal of a phosphate from tyr^{527} at the C-terminal tail, or by mutations within the SH2 domain. An intramolecular interaction between these two domains in the intact molecule may thus provide a mechanism to maintain c-Src in an inactive state. We have studied this proposed interaction using an agarose-immobilized synthetic peptide corresponding to the phosphorylated c-Src tail and the SH2 domain isolated as a bacterial glutathione S-transferase (GST) fusion protein. We demonstrate that the phosphorylated tail peptide binds to the SH2 domain with high affinity; this binding is inhibited by excess free phosphorylated tail peptide but not by the unphosphorylated peptide or phosphotyrosine. A mutant form of the GST-SH2 fusion with $148^{W\text{-}R}$, a mutation which activates the tyrosine kinase and transforming potential of c-Src (O'Brien, *et al*, 1990. Mol. Cell. Biol. 10:2855-2862), has greatly reduced binding towards the phosphorylated c-Src tail.

Purified GST-Src SH2 fusion protein can disrupt a complex in cell lysates of v-*Src* transformed chicken embryo fibroblast (CEF) that contain v-Src and a PI-3' kinase activity. Furthermore, the GST-Src SH2 fusion protein can bind PI-3' kinase in a lysate of v-Src transformed CEF but not parental CEF, suggesting that modification of the PI-3' kinase or a related protein (e.g. by tyrosine phosphorylation) is required for this association. The possible involvement of Src SH3 domain in these interactions is currently under study and the results will also be presented.

H 332 **STRUCTURE-FUNCTION STUDIES OF P21RAS GTPASE ACTIVATING PROTEIN (GAP),** George A. Martin, Robin Clark and Frank McCormick, Cetus Corporation 1400 Fifty-third Street Emeryville, CA 94608

The p21ras GTPase Activating Protein (GAP) can form stable complexes with cellular phosphoproteins mediated by the adjacent Src Homology Regions of GAP. These associated proteins include activated growth factor receptors and two phosophoproteins of 62 kD and 190 kD apparent molecular weight. We have constructed a series of deletion mutants of GAP and expressed them at high levels in insect cells using the baculovirus expression system. We are using these recombinant proteins to study the requirements and functional consequences of these interactions as well as a possible role of p21ras in forming these complexes in a variety of assay systems.

H 333 SH2 DOMAINS OF SIGNALING PROTEINS CONTROL SPECIFICITY OF BINDING TO GROWTH FACTOR RECEPTORS. Jane McGlade, Christine Ellis, Michael Reedijk, Geraldine Mbamalu, and Tony Pawson. Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave., Toronto, Ontario, Canada M5G 1X5.

The binding of cytoplasmic signaling proteins such as phosphatidylinositol (PI) 3'-kinase, phospholipase C-γ1 (PLC-γ1) and Ras GTPase activating protein (GAP) to autophosphorylated growth factor receptors is directed by their non-catalytic Src homology (SH) 2 domains. Different receptor tyrosine kinases complex with distinct sets of SH2-containing signaling proteins, suggesting that each receptor may activate an individual group of signal transduction pathways and evoke a unique cellular response. Within the platelet-derived growth factor receptor (PDGFR) subfamily, the activated βPDGFR associates with PI 3'-kinase, PLC-γ1 and GAP, whereas the closely related macrophage colony stimulating factor (CSF-1R) binds strongly to PI 3'-kinase in vivo, but poorly to PLC-γ1 or GAP. The Kit receptor tyrosine kinase, in contrast, associates with PI 3'-kinase and PLC-γ1 in response to Steel factor, but not with GAP. Bacterially expressed SH2 domains derived from the p85α regulatory subunit of PI 3'-kinase, PLC-γ1 and GAP bound to the βPDGFR, CSF-1R and Kit with the same specificity in vitro as displayed by the native signaling proteins in vivo. The specificity with which signaling proteins complex with different members of the PDGFR subfamily is therefore dictated by the sequences of their SH2 domains. We infer that the SH2 domains of p85, GAP, and PLC-γ1 bind to distinct high affinity sites on these receptors, whose creation is dependent on receptor autophosphorylation. These results suggest that the specificity with which SH2 domains bind to autophosphorylated PDGFR, and related receptors, is important in determining the activation of distinct signal transduction pathways in response to growth factor stimulation.

H 334 PARTIAL PURIFICATION AND CHARACTERISATION OF A PHOSPHATIDYLCHOLINE SPECIFIC PHOSPHOLIPASE D, Murray McKinnon and Peter J. Parker, Protein Phosphorylation Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX.

In response to various Ca^{2+}-mobilising agonists, many cells produce diacylglycerol (DAG) in a biphasic manner. There is now considerable evidence that Phosphatidylcholine (PtdCho) hydrolysis by either Phospholipase C or Phospholipase D/Phosphatidate phosphohydrolase is responsible for the secondary, sustained phase of DAG. In an attempt to further our understanding of this second messenger generating pathway, we have partially purified and characterised a PtdCho specific Phospholipase D activity from a particulate fraction of bovine brain.

H 335 ANALYSIS OF PLC-γ1 IMMUNOCOMPLEXES REVEALS KINSHIP OR CONFEDERATION OF PLC-γ1 WITH 3 CELLULAR PHOSPHOPROTEINS, Jill Meisenhelder and Tony Hunter, MBVL, Salk Institute, La Jolla, CA 92037

Three phosphoproteins of 47, 84 and 100 kDa are detected specifically together with phospholipase C-γ1 (PLC-γ1) in immunoprecipitates from NIH3T3 cell lysates made using certain classes of the monoclonal antibodies (MAbs) developed in S.G.Rhee's laboratory against bovine brain PLC-γ1. The fact that the 47 kDa protein is detected by Western blotting and immunoprecipitation from totally denatured lysates suggests that the 47 kDa protein is immunologically related to PLC-γ1 rather than being an associated protein. In contrast, glycerol gradient analysis and peptide mapping data indicate that the other two proteins form a complex with PLC-γ1. To determine what may be the sites of interaction between PLC-γ1 and the 84/100 kDa proteins and/or the epitope shared by p47 and PLC-γ1, the epitopes of different MAbs were mapped to specific regions of PLC-γ1. This was accomplished by synthesizing short fragments of PLC-γ1 in a reticulocyte lysate translation system; each MAb was then tested for its ability to immunoprecipitate these pieces. In this way the epitope of MAb B-6-4 (Group II by S.G.Rhee's classification scheme) was mapped to a 26 amino acid region at the N-terminal end of PLC-γ1. MAbs from four other groups all mapped to a 100 amino acid stretch of PLC-γ1 that includes the C-terminal half of the second SH2 domain, the two tyrosine kinase receptor phosphorylation sites, Y771 and Y783, and part of the SH3 domain. As some of these MAbs (Groups IV and V) coprecipitate these three phosphoproteins while others (Groups I and VI) do not, their epitopes were further defined by shotgunning 60 bp fragments of the cDNA encoding this region into an expression vector. Protein expression was induced in bacterial colonies on filters which were then screened with the individual MAbs in Western format. The results of this screening will be presented. The identity of the 47 kDa protein is being investigated by isolation and sequencing of cDNA clones selected from an expression library with MAbs that recognize the 47 kDa protein.

H 336 SENSITIVITY OF *ras*-RESPONSIVE ENHANCER ELEMENTS TO c-*raf*, Brett P. Monia, Nicholas M. Dean, C. Frank Bennett, Joseph F. Johnston, ISIS Pharmaceuticals, 2280 Faraday Ave, Carlsbad, CA 92008.

Three members of the *raf* gene family, designated c-*raf*-1, A-*raf* and B-*raf*, have been identified to date. Each of these genes has been shown, based on both structural and biochemical criteria, to encode highly conserved protein-serine/threonine kinases found principally as soluble cytoplasmic proteins. Specific alterations in *raf* gene structure leading to the expression of truncated *raf* gene products have been identified. These mutations convert normal *raf* genes to active oncogenes. Recent studies have suggested a role for the *raf* kinases as downstream effectors in signal transduction pathways initiated by the activity of plasma membrane oncogenes whose action leads to alterations in gene expression.

ras genes acquire transformation-inducing properties by single point mutations within their coding sequences. *ras* oncogene products bind guanine nucleotides, have GTPase activity and are associated with the inner surface of the plasma membrane. Expression of *ras* oncogenes in cells has been shown to result in alterations in expression of specific genes at the transcriptional level. Although the downstream effectors by which *ras* action on gene regulation have not been identified, c-*raf* has been implicated for such a role.

In this study, we are exploring the role of c-*raf* as a downstream effector of *ras*-mediated alterations in gene expression. Plasmids have been constructed containing different *ras*-responsive enhancer sequences each linked to the luciferase reporter gene and their sensitivity to expression of c-*raf* in transient transfection assays is being determined. Furthermore, the role of *raf*-mediated protein phosphorylation during gene induction by *ras* is being addressed. Results from these studies will be presented.

H 337 PHOSPHORYLATION-DEPENDENT SH2-BINDING BY rasGAP-ASSOCIATED PROTEINS, AND A NOVEL GNRF cDNA FROM MOUSE, Michael F. Moran, Vivien Measday, Luping Chen, Li Jia Wang, and Debra Gawler, Banting and Best Department of Medical Research, and Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario Canada M5G 1L6.

Activation of a variety of protein-tyrosine kinases results in the tyrosine and serine phosphorylation of rasGAP and the rasGAP-associated proteins p62 and p190. p62 and p190 bind to the SH2-containing region of rasGAP, and phosphosphorylation of p62 and p190 is required for SH2-binding. Bacterially expressed phosphotyrosine and phosphoserine phosphatases are being used to assess the role of individual phosphoamino acids in SH2-binding. In separate experiments, a putative guanine nucleotide release factor (GNRF) that has homology with the *S. cerevisiae CDC25* gene product has been molecularly cloned from mouse embryonic stem cells. Experiments are underway to characterize this cDNA and to identify associated GNRF activity.

H 338 STRUCTURAL ANALYSIS OF THE GENE ENCODING P21^{ras} GTPASE ACTIVATING PROTEIN (GAP), Janelle A. Noble*, George A. Martin, Mary Bennett and Frank McCormick, Cetus Corporation, 1400 53rd St., Emeryville, CA 94608 *Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

GAP (GTPase activating protein) is a 120 kd cytosolic protein which is expressed in all mammalian cells tested. GAP downregulates the activity of p21^{ras} by stimulating GTP hydrolysis. GAP interacts with other cellular proteins; functional consequences of these interactions are currently under investigation. We are determining the structure of the human gene encoding GAP. Southern blot analysis indicates that GAP is encoded by a single gene. We have isolated two non-contiguous λ genomic clones which include approximately 30 kb of genomic DNA. One of these clones contains the 3' end of the gene while the other clone contains the exons encoding the second of two SH2 domains present in the protein. PCR amplification of genomic DNA has allowed us to isolate an additional ~10 kb of genomic DNA containing GAP exons. With the exception of the final exon, which contains the 3' untranslated sequence and is >1kb in length, all exons mapped so far are small, ranging in size from ~50 to ~160 bp. We are currently utilizing both traditional cloning and PCR amplification cloning techniques to elucidate the structure of the remaining portion of the gene.

H 339 REGULATION OF TPA-INDUCED RESPONSES IN NIH3T3 CELLS BY GAP, THE GTPASE ACTIVATING PROTEIN ASSOCIATED WITH p21$^{c\text{-}ras}$, Mukund Nori, Gilles L'Allemain and Michael J. Weber, Department of Microbiology, Box 441, University of Virginia School of Medicine, Charlottesville, VA 22908

Proteins of the *ras* family of oncogenes have been implicated in signal transduction pathways initiated by several growth factors and the tyrosine kinase oncogene *src*. The activity of ras proteins has been shown to be regulated by a cellular protein, GAP (GTPase Activating Protein), that enhances the GTPase activity of p21$^{c\text{-}ras}$. In this paper, we report that overexpression of GAP results in an early blockage of activation of p42-Mitogen Activated Protein Kinase (p42^{mapk}) and inhibition of DNA synthesis triggered by tetradecanoyl phorbol acetate (TPA). Not all biochemical events elicited by TPA were affected, as increased glucose uptake and phosphorylation of MARCKS occurred normally. Reduction of GAP expression to near normal levels restored the ability of the cells to activate p42^{mapk} in response to TPA; however, induction of DNA synthesis by TPA remained blocked. These findings suggest that *ras*/GAP together play a key role in a PKC-dependent signal transduction pathway leading to the activation of p42^{mapk} and control of cell proliferation.

H 340 ACTIVATION OF RAS PROTEINS IN MAMMALIAN CELLS: INVOLVEMENT OF RAS P21 IN EGF SIGNAL TRANSDUCTION PATHWAY.

Fabienne PARKER, Anita DUGUE, Didier LANDAIS, Fabien SCHWEIGHOFFER Marc DUCHESNE and Bruno TOCQUE.

RHONE POULENC RORER - Centre de Recherche de Vitry-Alfortville-13, Quai Jules Guesdes - BP 14 - 94403 VITRY-SUR-SEINE CEDEX.

The proto oncogene ras acts as a component of signal transduction pathways in many kinds of cells. The activity of the ras gene product is controlled by bound GDP/GTP. In Vivo, ras p21s exist predominantly in their resting state, in a GDP bound form and become activated when an as yet unknown uptream signal promotes the transition p21-GDP -> p21-GTP. This transition is observed in vivo when cells are stimulated by several different growth factors including EGF, PDGF, and by oncogene products with enhanced tyrosine kinase activities. In this report we demonstrate the involvement of ras p21 in signal transduction from EGF but not from thrombin. Increases in GTP bound active ras p21 were observed in chinese hamster lung fibroblasts (CCL39) overexpressing human EGF-R (ER22) after addition of EGF but not after addition of thrombin, another potent mitogen for this cell line. These results strongly suggest that ras p21 is involved in signal transduction from tyrosine kinase growth factor receptors but not from G protein coupled receptors. A model to explain the EGF activity would be to envision either an inactivation of GTP-ase Activation Protein such as GAP-ras or NF1 or a devict activation of an Exchange factor for ras protein. To test these different hypothesis 3H-thymidine incorporation in ER22 cell line was measured after expression of plasmids in transient experiments.
Expression of the C-terminus domain but not the full length of GAP-ras blocks EGF-dependent loading of GTP onto ras and partially EGF induced DNA reinitiation.
Expression of the same plasmids does not impairs thrombin activity.

H 341 p120 GAP MAY MEDIATE INSULIN-INDUCED p21*ras* ACTIVATION. G.J. Pronk[1], R.H. Medema[1], B.M.Th. Burgering[1], J.A. Maassen[2], A.M.M. de Vries-Smits[1], F. McCormick[3] and J.L. Bos[1]. [1]Laboratory for Physiological Chemistry, University of Utrecht, Utrecht, The Netherlands; [2]Department of Medical Biochemistry, Sylvius Laboratory, Leiden, The Netherlands; [3]Cetus Corporation, Emeryville, California, USA.

Mitogenic responses induced by PDGF and EGF can be blocked by micro-injection of neutralizing p21*ras* antibodies. Also, p120 GAP is phosphorylated on tyrosine residues by a number of activated protein tyrosine kinases, such as the PDGF- and EGF-receptors. Therefore, both p21*ras* and p120 GAP seem to be involved in the transduction of signals from these activated receptors. Recently, we showed that p21*ras* is activated when NIH 3T3 cells overexpressing insulin receptors (A14 cells) are stimulated with insulin (Burgering et al., EMBO J. 10, 1991, p.1103). Furthermore, in transient transfection assays we could block insulin-induced c-fos promoter activation with dominant inhibitory mutants of p21*ras* . From these results, we conclude that p21*ras* is involved in insulin-induced signal transduction.
To investigate the possible mechanism by which insulin can activate p21*ras*, we used phenylarsine oxide (PAO), a putative phosphatase inhibitor that can block certain insulin-induced effects and reveal potential substrates for the insulin receptor kinase. In the A14 cells, PAO blocks insulin-induced p21*ras* activation. Furthermore, PAO revealed an insulin dependent tyrosine phosphorylation of p120 GAP, suggesting some kind of interaction between p120 GAP and the insulin receptor.
From these results, a model emerges in which insulin-induced p21*ras* activation is mediated through p120 GAP.

H 342 THE INTERACTION OF BCR-ABL ONCOPROTEINS WITH SH2 CONTAINING PROTEINS. Lorri Puil and Tony Pawson. Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave., Toronto, Canada M5G 1X5

In Philadelphia chromosome positive (Ph'+ve) leukemias, oncogenic activation of *c-abl* is acheived by fusion with 5' sequences of the *bcr* gene. A 210 or 185 kDa bcr-abl fusion protein is produced that exhibits deregulated tyrosine kinase activity. The Src Homology 2 (SH2) domain, originally described in non receptor tyrosine kinases, is a conserved motif that binds with high affinity to tyrosine phosphorylated ligands, and is found in a diverse group of signalling and transforming molecules including $p21^{ras}$ GAP, PLC-γ and the p85 subunit of PI3'-kinase. We investigated whether GAP and other SH2 containing proteins are intracellular targets of bcr-abl and examined the nature of their interaction with the bcr-abl oncoproteins. In rat fibroblasts infected with retroviral vectors expressing p185 or $p210^{bcr\text{-}abl}$, and in human cell lines derived from patients with CML or Ph'+ve ALL, GAP forms a stable complex with bcr-abl and is phosphorylated on tyrosine. Using bacterially expressed fusion proteins, we demonstrated that bcr-abl binds specifically to the SH2 domains of GAP *in vitro*. Other SH2 containing proteins bind with varying efficiency to bcr-abl, also by virtue of their SH2 domains. Studies to determine the contribution of bcr and abl derived sequences to the interaction with these proteins, and the role of phosphorylation, will be presented.

An additional phosphoprotein that is associated with GAP in cells transformed by tyrosine kinases is a 62 kDa protein, p62. In human leukemic cell lines and primary blast cells expressing $p185^{bcr\text{-}abl}$, GAP-associated p62 is a predominant tyrosine phosphorylated protein. p62 is capable of binding to both GAP and abl SH2 domains *in vitro*. In fibroblasts, p62 co-migrates with a major phosphoprotein previously reported to correlate with the transforming ability of $p185^{bcr\text{-}abl}$ deletion mutants (T. Lugo *et al* (1990). Science 247, 1079). These observations suggest that GAP-associated p62 is a potentially critical substrate in Ph'+ve leukemia, and that a network of interactions involving the SH2 domains of signalling molecules occurs in cells expressing bcr-abl.

H 343 A TYROSINE KINASE ACTIVITY IS LATELY ACTIVATED UNDER IL1 STIMULATION OF HUMAN LYMPHOCYTE. Didier MARY and Bernard ROSSI, Unité INSERM 210, Faculté de Médecine Pasteur, Nice 06034, FRANCE

IL1 delivers, in conjunction with TcR triggering, a costimulatory signal which leads to cell activation and eventually to lymphocyte proliferation. This effect is mediated though a Mr = 80 kDa surface receptor. Analysis of the sequence of the receptor cytoplasmic domain provides very few clues as to the post receptors events might be. The only few information related to IL1 signal transduction concerns a rapid release of DAG from a pool of phosphatidylcholine by activation of a phospholipase C distinct from that involved in the phosphoinositides cleavage. These data support the hypothesis of a participation of the protein kinase C in the transduction of the IL1 signal. No evidence of the involvement of a tyrosine kinase in the IL1 signal transduction have been advanced as far.

Here, we provide evidence showing that a tyrosine kinase activity is involved in the propagation of the activating IL1 signal inside the cell. In a first experiment, proteins phosphorylated on tyrosine residues were visualized by a two dimensional separation on polyacrylamide gel, followed by an alkali treatment, using Jurkat cells preloaded with 32P inorganic phosphate. Under IL1 stimulation we observed a complex pattern of phosphorylation. A first set of high molecular weight proteins (> 130 kDa), a second set of proteins travelled with an apparent molecular weight of 80-100 kDa, two additional other bands migrated with an apparent Mr of 66 and 20 kDa, respectively. Western blot, using anti-phosphotyrosine antibodies confirmed that proteins with a Mr of 150, 100, 85 and 66 kDa, respectively, were phosphorylated on tyrosine residues. It is noteworthy that with this technique pp20 was not detectable. However the presence of phosphotyrosine residues on this protein was verified by phosphoaminoacid analysis.

Activation of this tyrosine kinase activity is very peculiar in the sense that it peaks several hours after exposure of Jurkat cells to IL1α or IL1β. Besides to appear lately, the IL1-induced tyrosine kinase is transient, with an activation phase of about 2 hours.

H 344 CHARACTERIZATION OF THE GTPase ACTIVATING PROTEIN SPECIFIC FOR THE rap-1/k-rev-1 PROTEIN. Bonnee Rubinfeld, Iris Albert, Leah Conroy, Robin Clark, Heinz Haubruck, Frank McCormick and Paul Polakis. Department of Molecular Biology, Cetus Corporation, 1400 Fifty-third Street, Emeryville, CA 94608.

rap1GAP is a protein that specifically stimulates the GTP hydrolytic rate of the ras-related protein, $p21^{rap1A}$. Recently we reported the cloning and expression of two human cDNAs encoding rap1GAP. In order to characterize the functional domains of rap1GAP, we constructed a series of deletion mutants involving progressive truncations at the amino-and carboxyl-termini. These mutants were engineered to encode a nine amino acid antibody epitope tag when expressed in the baculovirus system. These additional amino acid residues had no detectable effect on rap1GAP activity and allowed us to affinity purify the mutant rap1GAP proteins for testing. Mutational analysis narrowed the catalytic domain of rap1GAP to the amino-terminal portion of the protein. While the carboxyl terminal portion of rap1GAP is dispensible for GTPase stimulating activity, it contains sites of phosphorylation for both cAMP-dependent kinase and the cell cycle cdc2 kinase. Purified rap1GAP was phosphorylated by both these kinases in vitro. Phosphopeptide analysis suggested that the same sites are phosphorylated in vivo. Interestingly, these sites of phosphorylation lie within a region of rap1GAP that is duplicated as a result of an mRNA splicing event.

To determine the subcellular location of rap1GAP, immunofluorescence experiments were carried out using the SK-MEL-3 cell line as well as rat-1 cells stably expressing the rap1GAP cDNA. In both cases, specific antibodies localized rap1GAP to the Golgi apparatus. The localization of the deletion mutants will also be discussed.

H 345 *CSK* INDUCED REVERSION OF RAT 3Y1 CELLS TRANSFORMED BY COEXPRESSION OF C-*SRC* AND V-*CRK*. Hisataka Sabe*, Masato Okada#, Beatrice Knudsen*, Shigeyuki Nada#, Hachiro Nakagawa#, and Hidesaburo Hanafusa*, *Laboratory of Molecular Oncology, The Rockefeller University, New York, NY 10021, #Division of Protein Metabolism, Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan

The regulation of c-Src kinase activity by phosphorylation of Tyr527 has been well characterized. Recently, CSK (C-terminal Src Kinase) was isolated as a potential kinase that phosphorylates c-Src Tyr527, and its cDNA was isolated from a rat brain library. More recently, we isolated chicken *csk* cDNA and showed that amino acid sequences of rat and chicken CSK proteins are highly conserved (93%). To analyze the biological activity of CSK in vivo the following experiments were performed. Overexpression of v-*crk* resulted in increase in the level of phosphotyrosine in rat 3Y1 cells but cellular transformation was barely detectable. Likewise, expression of c-*src* alone caused no transformation in rat 3Y1 cells. However, coexpression of these two genes induced discernible morphological changes in a significant fraction of cells. We found that expression of *csk* can induce reversion of morphology in the majority of these cells. The results strongly suggest that CSK indeed serves to regulate the c-Src kinase activity in vivo. The molecular mechanism for the interaction among c-Src, v-Crk, and CSK will be discussed.

H 346 GROWTH FACTOR-DEPENDENT ACTIVATION OF RAS IS MEDIATED BY TYROSINE KINASES, Takaya Satoh, Masato Nakafuku, Tomokazu Ohtsuka, Atsushi Miyajima, and Yoshito Kaziro, DNAX Research Institute, Palo Alto, CA 94304

Ras protein acts as a transducer of signals controlling cell growth and differentiation, and its activity is regulated by the species of bound guanine nucleotide (GDP vs GTP). Since the increase of active GTP-bound form reflects the activation of Ras protein, we measured the Ras-bound GDP/GTP in the cell following the stimulation by various growth factors and lymphokines. PDGF and EGF, which induce the DNA synthesis in quiescent fibroblast cells through receptors with tyrosine kinase activity, were found to accumulate an active GTP-bound form of Ras. The increase of Ras·GTP was detected within one minute after the addition of the growth factors. We also detected high levels of Ras·GTP in transformants carrying Src or ErbB-2/Neu oncoproteins whose tyrosine kinases are constitutively active. These results suggest that Ras protein is regulated by receptor and non-receptor tyrosine kinases in signal transduction pathways of cell growth and transformation. In hematopoietic cells, GTP-bound Ras increased after the stimulation with IL-2, IL-3, and GM-CSF. Although receptors for these lymphokines have no intrinsic tyrosine kinase activity, non-receptor type tyrosine kinases may play an important role in the signal transduction. We found that a specific inhibitor of tyrosine kinases, herbimycin A, completely blocked the increase of Ras·GTP by IL-3 or GM-CSF, suggesting the involvement of a tyrosine kinase in the process of Ras activation by these lymphokines.

H 347 EXPRESSION OF *n*-CHIMAERIN, A NEURONAL P21*rac* GTPASE ACTIVATING PROTEIN AND PHORBOL ESTER RECEPTOR. Wun Chey Sin[§†], Hong Hwa Lim[§†], Gregory Michael[§†], Christine Hall[§] & Louis Lim[§†] [§] Department of Neurochemistry, Institute of Neurology, 1 Wakefield Street, London WC1N 1PJ; [†] Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511.

n-Chimaerin is a GTPase activating protein (GAP) for p21*rac* and a phorbol ester/diacylglycerol receptor. Human and rat *n*-chimaerin cDNA sequences are highly conserved (97% overall amino acid identity), with precise conservation of the protein kinase C-like cysteine-rich motif involved in phorbol ester binding. The C-terminal regions of *n*-chimaerin and BCR (the breakpoint cluster region gene product) are sequence related and both have GAP activity.

Isolation of the rat cDNA elucidated a sequence inversion of the human cDNA 5'UTR, extending the predicted N-terminal of the protein by a 35 amino acid sequence with a predicted amphipathic helical structure. 5' RACE PCR of rat brain *n*-chimaerin mRNA provided evidence for 5' sequence heterogeneity. A variant *n*-chimaerin cDNA encodes a potential receptor binding domain in an alternate N-terminal sequence. *n*-Chimaerin mRNA is specifically expressed in neurones, with high levels in the hippocampus and cortex and in the cerebellum it is restricted to Purkinje neurones. The specificity of mRNA expression and its localization to brain regions implicated in learning and memory processes suggest an important role for *n*-chimaerin as a GAP involved in neuronal signal transduction mechanisms.

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

H 348 **EXPRESSION OF BCR-ABL IN HUMAN HEMATOPOIETIC CELLS ABROGATES FACTOR DEPENDENCE BY AN AUTOCRINE MECHANISM AND ALTERS THE PROLIFERATION AND DIFFERENTIATION PROGRAM.** Christian Sirard, Tsvee Lapidot and John Dick, Dept. of Genetics, Research Institute, Hospital for Sick Children and Dept. of Molecular and Medical Genetics, University of Toronto, Ontario, Canada.

Evidence has accumulated to support the hypothesis, originally proposed by Sporn and Todaro, that autocrine stimulation is a mechanism involved in the multi-step process of neoplastic growth. Leukemic cells from several human lymphoid and myeloid leukemias show increased expression of growth factor genes to which they respond. In AML, growth autonomy has been attributed to genetic rearrangement of growth factor genes. In murine and avian myeloid leukemia, growth autonomy can be caused by viral oncogenes such as v-sis, v-src-family or myc/mil genes. However, no studies have demonstrated the involvement of oncogenes in autocrine stimulation of human leukemic cells. We have introduced the bcr-abl cDNA, by retroviral infection, into a human factor dependent cell line, MO7E. This cell line was derived from a patient with megakaryocytic leukemia and requires the presence of GM-CSF, IL-3 or the c-kit ligand for its proliferation. Factor independent (FI) populations were readily obtained in MO7E cells expressing a functional $p210^{bcr\text{-}abl}$. Cytochemical stains revealed that the bcr-abl expressing cells became further differentiated along the megakaryocytic pathway. Induction of GM-CSF and IL-3 message was detected in the FI population by RT-PCR. In addition, the FI cells remained responsive to those exogenous factors, suggesting that an autocrine loop was responsible for their growth autonomy. Evidence for the secretion of a stimulatory factor was obtained by growing MO7E cells either in presence of irradiated FI cells or in presence of concentrated FI conditioned media. This activity was partially inhibited with anti-GM-CSF antibody and completely neutralized when used in combination with anti-IL-3 antibodies. These results establish for the first time that the expression of an oncogene, in human hematopoietic cells, can lead to growth autonomy via an autocrine mechanism. Furthermore, expression of bcr-abl also considerably reduced the generation time of M07E cells in vitro. This proliferative advantage of the FI cell population was also shown to to take place in vivo using an immune-dedicient scid mouse model.

H 349 PURIFICATION AND CHARACTERIZATION OF PTDINS (4)*P* 5-KINASE FROM BOVINE BRAIN, Catherine E.L. Spencer, Nullin Divecha and Robin F. Irvine, Department of Biochemistry, AFRC Institute of Animal Physiology and Genetics Research, Babraham Hall, Cambridge, CB2 4AT, U.K.

Following receptor activated stimulation of phosphoinositidase C (PIC) there must be an activation of PtdIns (4,5)P_2 synthesis in order to maintain sufficient substrate for the continued production of the second messengers Ins (1,4,5)P_3 and 1,2,diacylglycerol. The mechanisms controlling the concentration of PtdIns (4,5)P_2 available to be hydrolysed by PIC are not known, but recent research has focused on PtdIns (4)*P* 5-kinase as an important regulatory enzyme in this respect.

As a preliminary step towards investigating its regulation, we have purified three isoforms of PtdIns (4)*P* 5-kinase from bovine brain cytosol, and shown them to be kinetically distinct. The 53kD enzyme is immunologically indistinguishable from the type 2 isozyme purified from human erythrocytes by Bazenet *et al.* There are also 110kD and (possibly) 45kD species which are not recognized by a polyclonal antibody to the 53kD protein. Investigations of the tissue distribution of these enzymes reveal clear tissue specificities, which suggest that PtdIns (4)*P* 5-kinase is a family of enzymes with distinct physiological properties and functions.

H 350 ACTIVATION OF A $p34^{cdc2}$-RELATED HISTONE H1 KINASE BY TYROSINE PHOSPHORYLATION IN v-*src* TRANSFORMED FIBROBLASTS, David W. Sternberg, Yasuhisa Fukui, and Hidesaburo Hanafusa, The Rockefeller University, New York, N.Y.

Transformation with oncogene tyrosine kinases or growth factor stimulation have been shown to activate many serine/threonine kinases. To identify kinases that are directly activated by the $pp60^{v\text{-}src}$ tyrosine kinase, we have used anti-phosphotyrosine immunoaffinity chromatography to enrich for candidate serine/threonine kinases that are modulated by protein-tyrosine phosphorylation. Lysates from the rat fibroblast line 3Y1 or the v-*src* transformed derivative SR-3Y1 were incubated with immobilized PY20 antibody, a monoclonal antibody directed against phosphotyrosine residues, and bound proteins were eluted with phenyl phosphate. The phosphorylation of exogenously added histone H1 was elevated 20-fold in the SR-3Y1 PY20 eluate compared to that of the 3Y1 control, and the histone H1 substrate was phosphorylated exclusively on serine residues. Treatment of the SR-3Y1 PY20 eluate with the catalytic domain of the LAR tyrosine phosphatase inactivated the histone H1 kinase, and this inactivation was completely blocked by incubation with phosphatase in the presence of vanadate and molybdate. These findings indicate that the specific activity of the histone H1 kinase is modulated by tyrosine phosphorylation in v-*src* tranformed fibroblasts. Fractionation of the SR-3Y1 PY20 eluate by MonoQ FPLC demonstrated a single peak of histone H1 kinase activity. Using a 3Y1 cell line transformed with a temperature-sensitive mutant of v-*src*, PY20 eluates were prepared from cells maintained at temperatures permissive or non-permissive for transformation. Elevated histone H1 kinase activity was recovered in the PY20 eluate derived from cells shifted to the permissive temperature for 15 min., indicating that the phosphorylation of the histone H1 kinase is an early event in v-*src* transformation. The histone H1 kinase activity was precipitated from SR-3Y1 PY20 eluates using $p13^{suc1}$-Sepharose beads, strongly suggesting that the histone H1 kinase is a member of the $p34^{cdc2}$ family of kinases.

H 351 HORMONAL REGULATION OF $cPLA_2$ Lisa A. Sultzman, Lih-Ling Lin, Alice Lin, James Clark, , Dina Martin, and John Knopf. Genetics Institute Cambridge Ma 02140

Thrombin, ATP, and the Ca^{2+} ionophore A23187 stimulate the rapid production of arachidonic acid in CHO cells. We have shown that following stimulation by these agents, CHO cells overexpressing $cPLA_2$ display an increased release of arachidonic acid as compared with the parental CHO cells, demonstrating that $cPLA_2$ is coupled to hormonally regulated release of arachidonic acid. Importantly, CHO cells overexpressing $sPLA_2$ fail to show increased hormonal responsiveness. We show that the stimulation of cells with thrombin, ATP, A23187, TPA, EGF, or PDGF results in the rapid phosphorylation of $cPLA_2$ on serine residues. The importance of the $cPLA_2$ phosphorylation was demonstrated in two ways: staurosporine inhibited both the ATP-stimulated $cPLA_2$ phosphorylation and arachidonic acid release, and the phosphorylated form of $cPLA_2$ showed increased activity in a liposome assay. Pertussis toxin (PTX) pretreatment has been shown to inhibit the ATP, thrombin and A23187 stimulated release of arachidonic acid. The effects of PTX pretreatment on the agonist induced phosphorylation of $cPLA_2$ and intracellular Ca^{2+} flux in CHO cells will be presented. Lastly, a series of mutagenesis experiments will be discussed further clarifying the role of the Ca^{2+} dependent Lipid Binding (CaLB) domain in $cPLA_2$.

H 352 Differential Cytotoxic Action of Lovastatin Against Epithelial Cells Transformed by Ras Oncogenes.

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Recent evidence indicates that ras protein is farnesylated near the carboxyl terminus and this modification is required for ras transforming activity. Lovastatin is a very potent inhibitor of HMG-CoA reductase activity in vitro (Alberts;PNAS; 77: 3957, '80) and can inhibit farnesylation of ras protein by decreasing the amount of farnesylpyrophosphate available for transfer. We have examined the cytocidal action of this drug against mouse mammary epithelial cells and cells transformed by Hras or Nras oncogenes. The LC_{50} of the parent cell line is between 3.5 and 25 times greater than the LC_{50} of Hras or Nras transformed cell lines. These results indicate the possibility that drugs that affect farnesylation may be useful anticancer drugs against tumors that contain an activated Hras or Nras oncogene.

H 353 HRAS INDUCED CARDIAC CELL HYPERTROPHY

Andrew Thorburn[1], Jackie Thorburn[2], Scott Powers[3], James R. Feramisco[1,2] and Kenneth R.Chien[2].
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We have investigated the role of the proto-oncogene HRas in cardiac cell growth and hypertrophy. We find by a combination of needle microinjection and transfection techniques that active Ras protein is able to stimulate the hypertrophic response in rat neonatal ventricular cardiac cells.In particular we show by microinjection that an oncogenic mutant Ras protein (Val 12) stimulates an immediate early gene response associated with hypertrophic agents in these cells, and induces expression of the Atrial Naturetic Factor (ANF) gene whose expression is a marker for hypertrophy. We also find that the ANF promoter is stimulated by Ras in a transfection assay and we demonstrate that expression from this promoter can be blocked by a dominantly acting negative Ras mutant (Ala 15), suggesting that the normal hypertrophic response in these cells is mediated through Ras. Experiments to further define the role of wild type Ras in this biological response are currently underway.

H 354 p21ras ACTIVATION IN AN ERYTHROPOIETIN SIGNAL TRANSDUCTION PATHWAY. M. Torti, K. Bencke Marti, D. Altschuler, K. Yamamoto and E.G. Lapetina. Division of Cell Biology, Burroughs Wellcome Co., Research Triangle Park, N.C. 27709

Erythropoietin (Epo), a 34 kDa glycoprotein, is the major regulator of the proliferation and differentiation of erythroid precursors. The chemistry and structure of this glycoprotein has been well characterized to allow its molecular cloning and expression; however its mechanism of action is poorly understood. Epo binds to a specific cell surface receptor that has a single transmembrane domain and shares sequence homology with the receptors for GM-CSF, IL-3, IL-4, IL-6 and IL-2. Very little is known about the molecular events following the interaction of the hormone with its receptor. In addition, the identity of second messenger(s) involved in the Epo signal transduction pathway is still controversial. For an increasing number of growth factors, including haematopoietic factors, an important role of the product of the protooncogene ras (p21ras) has been demonstrated. Using a human erythroleukemic cell line (HEL), we investigated whether p21ras is involved in Epo signal transduction. We found that stimulation of HEL cells with Epo induces a five-fold increase of the amount of GTP bound to the endogenous p21ras. This effect is dose dependent and occurs very rapidly, being detectable after 1 min from the addition of Epo. We also observed that Epo causes tyrosine phosphorylation of several proteins in a time-dependent manner, which correlates with p21ras activation. Moreover, inhibition of tyrosine kinases by genistein totally prevented Epo-induced accumulation of GTP-p21ras complex. Using an antiserum against the GTPase activating protein (GAP), we found that p120GAP is rapidly tyrosine phosphorylated in response to Epo. Furthermore, the ability of a lysate from Epo-stimulated HEL cells to induce 'in vitro' hydrolysis of GTP bound to p21ras was strongly reduced when compared with lysate from unstimulated cells. These results demonstrate that activation of p21ras is an early event occurring in the Epo signal transduction pathway and suggest that accumulation of GTP-p21ras complex may be triggered by the inhibition of the ras-GAP activity. These results also support the idea that tyrosine phosphorylation of p120GAP might affect its regulatory role on p21ras.

H 355 CALCIUM ION INFLUX ACTIVATES A RAS-RESPONSIVE ENHANCER ELEMENT. Massimo Ulivi and Michael C. Ostrowski, Department of Microbiology & Immunology, Duke University Medical Center, Box 3020, Durham, NC, 27710.

We are utilizing ras-responsive genes and the cis-acting enhancer elements that confer ras-responsiveness as targets in defining ras-signal transduction pathways. For example, we have recently shown that both ras and c-fms can activate the same nuclear targets and that overexpression of the catalytic domain of the GTPase activating protein (GAP) can suppress c-fms nuclear signal transduction. The data suggest that c-fms and ras are in a common signal transduction pathway. We now have evidence indicating that calcium ions may play a role in ras nuclear signal transduction. First, the calcium ionophore A23187 can activate the ras-responsive NVL-3 retrotransposon and the ras-responsive enhancer element located in the promoter sequences for NVL-3. Second, inhibitors of calcium ion influx, such as EGTA and nickel ions, block c-fms activation of NVL-3 in the presence of recombinant CSF-1. Third, a constitutive form of calmodulin-activated kinase can trans-activate the NVL-3 ras-responsive enhancer element in transient transfection assays. These data indicate that either ras and calcium ions are in a single signal transduction pathway, or that these agents represent distinct pathways that converge at common nuclear targets. Pharmacological, biochemical, and molecular genetic approaches are currently underway to distinguish between the alternative hypotheses.

H 356 *Abstract Withdrawn*

H 357 EARLY SIGNALS INVOLVED IN PLATELET-DERIVED GROWTH FACTOR STIMULATED MITOGENESIS OF 3T3 CELLS. Calvin C. Wilhide, III, Marilyn Webster, Robert W. Tucker, The Johns Hopkins Oncology Center, Baltimore, MD 21205.

The increased expression of immediate early genes induced by Platelet-Derived Growth Factor (PDGF) does not require protein synthesis and instead involves direct activation of gene expression by intracellular signals. To elucidate the signalling pathway for the initiation of early gene expression after PDGF (BB, 30 ng/ml) stimulation, inositol trisphosphate (IP_3) was measured by a competitive binding assay using IP_3-binding protein derived from rat cerebellum, diacylglycerol (DAG) was measured using a DGKinase assay for sn-1,2 diglyceride, and Ca_i transients were detected using digital image analysis of Fura 2-loaded cells. The induction of early genes c-fos, c-jun, jun-b, and 268 were measured by in situ hybridization with antisense ^{35}S-RNA probes of the respective genes and by Northern gels. We have previously reported that PDGF-stimulated DNA synthesis in quiescent BALB/c 3T3 cells requires phospholipase C (PLC) activation, since Quin2/AM inhibited both Ca_i increases and DAG production, and subsequent PDGF-stimulated DNA synthesis. Moreover, the induction of the early genes c-fos and jun-b, but not c-jun or 268, were also inhibited by Quin2/AM. Fos and jun dimers bind AP-1 sites on DNA, and contribute to stimulation of DNA synthesis. Evidently dimers involving only c-jun were insufficient to stimulate AP-1 activity. Ca_i increases alone (induced by calcium ionophore A23187) were also not sufficient to induce DNA synthesis or early gene activation, but could synergize with exogenous DAG (DiC8) to produce approximately 50% of the DNA synthesis stimulated by PDGF (% labeled nuclei). PDGF also induced a sustained increase in IP_3 that could contribute to prolonged calcium influx and subsequent DAG increase. Indeed, calcium ionophore can induce sustained increases in DAG. Thus, IP_3, calcium, and DAG form an interlocking set of early intracellular signals that contribute in important ways to the activation of the fos and jun gene families necessary for AP-1 activity and DNA synthesis. It is of particular interest that the induction of fos and jun proteins occurs via different intracellular signals.

H 358 TWO FORMS OF THE MURINE C-FGR PROTEIN TYROSINE KINASE ARISE BY ALTERNATIVE TRANSLATION INITIATION: pp59C-FGR AND pp53C-FGR HAVE DIFFERENT SUBCELLULAR LOCATIONS AND FUNCTIONS IN MONOCYTIC CELLS, Cheryl L. Willman, Jeffrey Potter, Walter Duran, and Tao-Lin Yi. Depts. of Cell Biology and Pathology, Univ. of New Mexico School of Medicine, Albuquerque, NM 87131.

The c-fgr protein tyrosine kinase, a member of the src family of non-receptor tyrosine kinases, is selectively expressed in myeloid cells. Our previous studies have determined that c-fgr may play an important role in the development and function of monocytic cells (PNAS 86:4254; Oncogene 4:1081; Blood 77:726). CSF-1, the monocytic lineage growth factor, induces a transient increase in the expression of c-fgr mRNA and protein, which peaks at the G1/S phase transition in monocytes (PNAS 86:4254). We have determined that the c-fgr cDNA, derived from CSF-1 stimulated murine bone marrow-derived macrophages (BMM) (Oncogene 4:1081) encodes two distinct proteins through alternative translation initiation. The full-length protein of 517 aa, pp59c-fgr, contains the unique c-fgr 70aa amino terminal domain, followed by the SH3, SH2, and kinase domains conserved in all src family members. Analysis of BMM and COS cells transiently transfected with several c-fgr constructs have revealed that a second c-fgr protein of 459aa arises through alternative translation initiation at an internal methionine residue at the end of the amino terminal domain. This smaller protein, pp53c-fgr, thus lacks myristylation signals and contains only the c-fgr SH3, SH2, and kinase domains. Affinity purified anti-peptide antisera specific for the unique c-fgr amino or carboxy terminal domains have been developed to study the subcellular locations and functions of these proteins: while pp59c-fgr is associated with plasma membrane at sites of focal adhesion plaques and in membrane ruffles, pp53c-fgr is localized to the nucleus and perinuclear endoplasmic reticulum in both transiently transfected COS cells and BMM. When BMM are synchronized and stimulated to proliferate with CSF-1, the levels of both pp59c-fgr and pp53c-fgr increase. However, these two proteins are differentially altered at the G1/S phase transition: pp53c-fgr, associated with the nucleus is altered to a slower migrating form (likely through phosphorylation), while pp59c-fgr associated with the plasma membranes remains unchanged. These studies suggest that these two c-fgr proteins have different functions as well as subcellular locations in monocytic cells.

H 359 GROWTH HORMONE STIMULATES THE TYROSINE PHOSPHORYLATION OF ERK-RELATED PROTEINS, Linda A. Winston and Paul J. Bertics, Dept. of Physiological Chemistry, University of Wisconsin, Madison, WI 53706.

We recently reported that growth hormone (GH) rapidly stimulates the tyrosine phosphorylation of a number of proteins in 3T3-F442A cells, a cell line in which GH is reported to promote differentiation and inhibit mitogen-stimulated growth. We have now identified two GH-stimulated phosphotyrosyl proteins, with molecular masses of 42kD (pp42) and 45kD (pp45), as being immunologically-related to the ERK1 protein. ERK1 co-purifies with a microtubule-associated protein 2 kinase activity, and can also phosphorylate myelin basic protein (MBP). Tyrosine phosphorylation of pp42 and pp45 was maximal by 10 min of GH treatment and declined to control levels by 30 min. Even at 10 min, however, GH-stimulated tyrosine phosphorylation of pp42 and pp45 was of lesser magnitude than that stimulated by mitogens such as PDGF and EGF. Preliminary evidence suggests that maximal levels of GH-stimulated tyrosine phosphorylation of pp42 correspond to peak levels of a 42kD renaturable MBP kinase activity in lysates of GH-treated cells resolved by SDS-PAGE. In contrast to an observed decrease in PDGF-stimulated tyrosine phosphorylation of pp42 and pp45 following 48 h phorbol ester pre-treatment, known to result in depletion of certain protein kinase C (PKC) isozymes, GH-stimulated tyrosine phosphorylation of these proteins was enhanced by phorbol ester pre-treatment. Thus, it would appear that GH stimulates tyrosine phosphorylation of pp42 and pp45 via a pathway that is largely distinct from that utilized by PDGF. Tyrosine phosphorylation of pp42 and pp45 is known to be stimulated by numerous mitogens; yet, stimulation of this activity by GH suggests that mitogens and non-mitogens, which initiate ultimately distinct cellular programs, may nonetheless employ common phosphotyrosyl proteins in their signalling pathways.

H 360 PROTEIN TYROSINE PHOSPHATASE CONTAINING SH2 DOMAINS: CHARACTERIZATION, PREFERENTIAL EXPRESSION IN HEMATOPOIETIC CELLS AND LOCALIZATION TO HUMAN CHROMOSOME 12P12-13.

T. Yi, J.L. Cleveland and J.N. Ihle, Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38101

Protein tyrosine phosphorylation has been implicated in the growth and functional responses of hematopoietic cells. Recently approaches have been developed to characterize the protein tyrosine phosphatases that may contribute to the regulation of protein tyrosine phosphorylation. One novel protein tyrosine phosphatase was predominantly expressed in hematopoietic cells. This hematopoietic cell phosphatase (HCP), encodes a 68 kDa protein that contains a single phosphatase conserved domain which is distinctly related to the known protein tyrosine phosphatases. Unlike other known protein tyrosine phosphatases, HCP contains two src homology 2 (SH2) domains. We also cloned the human homologue and show that it is highly homologous to the murine gene. Both the murine and human gene products have tyrosine specific phosphatase activity and both are predominantly expressed in hematopoietic cells. In IL-3-dependent cells, the growth factor had no marked effects on the steady state levels of transcripts for HCP. Importantly the human gene maps to chromosome 12 to the region p12-p13. This region is associated with rearrangements in approximately 10% of cases of acute lymphocyte leukemia in children. These data suggest that HCP may play an important role in signal transduction pathways in hematopoietic cells.

H 361 IDENTIFICATION OF S. POMBE GAP AND CAP.

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I have constructed a S. pombe cDNA library in a vector that can be used to express the cDNAs in S. cerevisiae from the ADH1 promoter. This library was screened for suppressors of the heat-shock sensitive phenotype induced by deletion of the IRA1 gene in S. cerevisiae. One clone identified from this screen encodes a S. pombe protein that is homologous to the S. cerevisiae IRA1 and IRA2 proteins and mammalian GAP protein. I have also identified S. pombe cDNAs that suppress phenotypes associated with deletion of the CAP gene in S. cerevisiae. CAP is an adenylyl cyclase associated protein with two distinct functional domains: the N-terminal domain appears to be required for proper regulation of adenylyl cyclase by RAS, while loss of the C-terminal region is associated with morphological and nutritional defects (1-3). One of the S. pombe cDNAs identified encodes a protein that shares significant sequence homology with CAP. This protein appears to be required for the proper function of S. pombe adenylyl cyclase and shares certain functions with S. cerevisiae CAP (4).

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3. Vojtek, A., B. Haarer, J. Field, J. Gerst, T. D. Pollard, S. Brown & M. Wigler (1991) Cell 66:497-505.
4. Kawamukai, M., J. Gerst, J. Field, M. Riggs, L. Rodgers, M. Wigler & D. Young (manuscript submitted)

H 362 CDC25 PROTEINS IN GROWTH REGULATION AND SIGNAL TRANSDUCTION IN YEAST AND MAMMALIAN CELLS Renata Zippel, Marco Vanoni, Paola Coccetti, Riccardo Brambilla, Cristina Ferrari, Lilia Alberghina, Emmapaola Sturani, Enzo Martegani. Dipartimento di Fisiologia e Biochimica Generali, Sezione di Biochimica Comparata, Università degli Studi di Milano

Ras proteins can cycle between an active (GTP-bound) and an inactive (GDP-bound) state. GTP hydrolysis and the exchange of the bound nucleotide are mediated by GTPase activating proteins (GAPs) and guanine nucleotide releasing proteins (GNRP) respectively. The prototype of the latter class of proteins is represented by the *Saccharomyces cerevisiae* *CDC25*-encoded protein.

We have isolated a mouse brain cDNA encoding a CDC25-like protein by direct functional complementation of a *cdc25-1* mutant. The 287 residue-encoded protein is 34 % identical with the catalytic carboxy terminal part of the CDC25 protein and shares significant homology with other proteins belonging to the same family, such as *S. cerevisiae* SDC25, LTE1 and BUD5, as well as with ste6 from *Schizosaccharomyces pombe*. The mouse cDNA specifically suppresses *cdc25* mutations and disruptions and restores - at least partially - the glucose-induced cAMP signal in a *S. cerevisiae* strain bearing a disruption of the *CDC25* gene.

We are currently investigating expression of the mouse *CDC25* gene in different tissues and cell lines. The phenotypic effects of controlled expression in both yeast and mammalian systems of the *CDC25* mouse cDNA and of a 5'-extended version we have recently isolated and that is fully functional in yeast will also be reported.

Signals and Differentiation

H 400 IDENTITY OF REARRANGED LINE/C-MYC JUNCTION SEQUENCES SPECIFIC FOR THE CANINE TRANSMISSIBLE VENEREAL TUMOR. E.N. Amariglio, I. Hakim, F. Brok-Simoni, Z. Grossman, N. Katzir, A. Harmelin, B. Ramot, G. Rechavi. Department of Hematology, The Chaim Sheba Medical Centre, Tel-Hashomer 52621, and Sackler School of Medicine, Tel-Aviv University, ISRAEL.

The canine transmissible venereal tumor (TVT) is a naturally occurring neoplastic disease that affects the external genitalia of both sexes and is transmitted during coitus. Cytogenetic and immunologic studies demonstrated that tumors from different parts of the world are very similar, suggesting that they are transferred from one animal to another by the transplantation of viable cells. We found that the C-MYC oncogene was rearranged in this tumor by the insertion of a transposable genetic element sequence (known as LINE -long interspersed element) 5' to the first exon .

The amplification of a DNA segment located in the junction of the LINE genome and C-MYC upstream sequences, enabled the testing of the similarity of TVT samples, independently collected in different parts of the world. Oligonucleotide primers flanking the LINE/C-MYC junction were used to amplify a 340 bp segment and nested primers amplified a 280 bp segment. A fifth oligonucleotide used as a probe contained the actual junction sequence. All the tumors analysed revealed the existence of the specific bands, which were absent in normal canine DNA samples. The amplified segments obtained from all the tumors analysed were identical both in size and nucleotide sequence, suggesting transmission of the original rearranged cell itself, as opposed to independent events of LINE insertion in a hot spot.

H 401 EFFECTS OF *MYC* ONCOGENE OVER EXPRESSION ON B LYMPHOID CELL DIFFERENTIATION, *Steven R. Bauer, #Richard H. Scheuermann, *Lab of Molecular Immunology, FDA 8800 Rockville Pike, Bethesda, MD. Department of Pathology, Southwestern Medical School, Dallas Texas.

We have established a series of transgenic mice which innapropriately express the *myc*-oncogene under the influence of wild-type or mutated versions of the immunoglobulin heavy chain intronic enhancer. Mice carrying the wild-type enhancer constructs succumb exclusively to B lineage tumors, primarily of mature B cell or late pre B cell phenotype. The mutated heavy chain enhancer constructs carry deletions of negative transcriptional regulatory elements, resulting in transformation of B lineage cells from very early as well as later B cell stages. These deletion constructs also result in transformation of T and myeloid lineage cells. We are characterizing the steps in early B cell differentiation by using a set of reproducible tumor phenotypes found in multiple founder strains from each construct. Among these reproducible tumor types are biphenotypic tumors which represent precursor-product transitions in B cell differentiation. Such results are particularly useful in studying early events in B cell differentiation. With a quantitative PCR technique, we are also measuring expression levels of several oncogenes in different tumor phenotypes which arise from the same initiating event of *myc* oncogene over-expression. We are also comparing the numbers of clonable preB cells in normal or pre-neoplastic mice to determine how early *myc* oncogene overexpression can perturb B lineage differentiation.

H 402 REGULATION OF THE NGF SIGNAL CASCADE BY N-myc, Emil Bogenmann, Frank Wu, Luo Feng, and Hiroshi Matsushima, Department of Pediatrics, University of Southern California School of Medicine, Childrens Hospital Los Angeles, Los Angeles, Ca. 90027.

Nerve growth factor (NGF) is an important neurotrophic factor responsible for the growth, differentiation and survival of sympathetic and neural crest derived sensory neurons. NGF binds to a membrane receptor which seems to be composed of two components, namely the $gp75^{NGFR}$ and the $gp140^{proto\text{-}trk}$. It is still controversial whether a functional NGF response requires both receptor chains.
We have investigated the regulation of the NGF signal cascade by the N-myc oncogene in a rat central nervous system derived cell line (B104), which does express the $gp75^{NGFR}$ gene but not the gene encoding the $gp140^{proto\text{-}trk}$. Transfection of the N-*myc* gene into B104 cells results in a 10 fold increase in the level of $gp75^{NGFR}$ mRNA. Similarly, a 10 fold increase in membrane bound $gp75^{NGFR}$ was determined by flow cytometry. Treatment of B104 and B104-N-*myc* cells with NGF does not activate immediate early genes, i.e. *c-fos*. Stable expression of the $gp140^{proto\text{-}trk}$ cDNA (kindly provided by M. Barbacid) in B104 cells stimulates cell growth when cells are treated with NGF. However, B104-N-*myc* cells expressing $gp140^{proto\text{-}trk}$ showed a substantial reduction in cell growth in the presence of NGF, suggesting cell differentiation. Scatchard analysis of ^{125}I NGF binding to B104 trk and B104-N-*myc* trk cell membranes demonstrates 2 binding site affinities ($Kd = 5\ 10^{-9}$ M, $Kd = 5 \times 10^{-11}$ M). However, the ratio of membrane bound $gp140^{proto\text{-}trk}$ to $gp75^{NGFR}$ in B104 trk cells is markedly different from $gp140^{proto\text{-}trk}$ cells. Future experiments will attempt to investigate whether the level of $gp140^{proto\text{-}trk}$ expression and/or the ratio of low affinity to high affinity receptor is a determining factor for mitogenesis versus differentiation.

H 403 A HUMAN KERATINOCYTE MODEL FOR THE MULTISTEP PROCESS OF CARCINOGENESIS
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As proposed for carcinogenesis in vivo malignant transformation of cultured human epidermal cells is a multistep process which can be subdivided into at least three distinct (genetically determined) stages: *immortalization - neoplastic transformation* (benign tumors) - *malignant conversion* (malignant tumors).
As a model we use human skin keratinocytes which immortalized spontaneously (HaCaT). The cytogenetic changes seen in the early passage (p 2) HaCaT cells involved chromosomes 3, 4 and 9 (monosomy of 3p, 4 and 9p and trisomy of 9q). Moreover, these immortal and non-tumorigenic HaCaT cells exhibit a high level of p53 upon Western blotting and histochemistry, suggesting the presence of the stable mutated form of p53.
Tumorigenicity did not occur spontaneously within 300 passages tested but could be induced by transfection with the c-Ha-ras oncogene. Hereby two distinct phenotypes appeared giving rise to a) benign and b) malignant tumors after s.c. injection, being non-invasive or invasive in the transplantation assay, respectively. The two phenotypes could neither be discriminated on the basis of p21 val-12 expression nor specific ras integration site. However, there was a clear difference in their sensitivity to TGFß and thus only the malignant cells were resistent or even became growth stimulated under in vitro conditions. Furthermore, when the ras oncogene containing chromosomes (micro nuclei transfer) were transferred into HaCaT cells of different passage levels, early passages ($<$p30) gave rise to benign tumors or remained non-tumorigenic while with later passage cells ($>$p30) 8 out of 9 clones developed malignant tumors. Thus, in addition to the expression of the ras oncogene, and most probably of the mutated form of p53, chromosomal changes present in the recipient cells (arising spontaneously during subsequent passaging) are apparently obligatory for malignant conversion of the HaCaT cells. How far this can be attributed to specific chromosomal losses or deletions is currently under investigation.

H 404 *Abstract Withdrawn*

H 405 GROWTH FACTOR ACTIVATION OF PHOSPHATIDYLINOSITOL 3-KINASE IN PC12 CELLS.
A.N.Carter and C.P.Downes, Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland.
Phosphatidylinositol 3-kinase (PI 3-kinase) phosphorylates the 3-position in inositol phospholipids to give the corresponding 3-phosphorylated compounds. The enzyme is activated by a handful of growth factors whose receptors possess tyrosine kinase activity. Platelet-derived growth factor, colony stimulating factor 1, and insulin all activate PI 3-kinase as measured by increases in the cellular levels of its products, phosphatidylinositol(3,4)P_2 and phosphatidylinositol(3,4,5)P_3, and by the growth factor dependent appearence of PI 3-kinase activity in antiphosphotyrosine immunoprecipitates. We have studied the activation of PI 3-kinase, as judged by the above criteria, in the rat pheochromocytoma cell line (PC12).These cells respond differentially to various growth factors; for example, nerve growth factor (NGF) induces differentiation to a neuronal phenotype, epidermal growth factor (EGF),is weakly mitogenic, and Insulin-like growth factors 1and 2 (IGF1 and 2), are both strongly mitogenic. Despite these distinct cellular endpoints we find that each of the above growth factors activates PI 3-kinase, but with different kinetics: PI 3-kinase activity peaked after 1 min of stimulation with EGF, after 5 mins with NGF, and 10 mins with IGF 1 and 2. The levels of phosphatidylinositol (3,4,5)P_3 increased up to 30-fold at its peak when it comprised approximately 5% of the level of phosphatidylinositol (4,5)P_2, substantially higher than in previous reports of growth factor effects on this lipid.We will discuss these data with respect to the idea that PI 3-kinase is a new effector in signal transduction, and the likelihood that one of its products is a new second messenger.

H 406 ACTIVATION OF A DNA-DEPENDENT PROTEIN KINASE IN VIRALLY TRANSFORMED HUMAN CELLS. Timothy H. Carter[1], William K. Kaufmann[2], Ivon Sun[1] and Sam Tang[1], [1]Dept. of Biological Sciences, St. John's Univ. NY 11439, and [2]Dept. of Pathology and Lineberger Comprehensive Cancer Center, Univ. of North Carolina at Chapl Hill, Chapel Hill, NC 27599-7295.

A protein kinase which requires the presence of double-stranded DNA (DNA-PK) was recently purified in several laboratories (Carter et al., Mol. Cell Biol. *10*, 6460, 1990; Lees-Miller et al., Mol. Cell Biol. *10*, 6472, 1990). Nuclear extracts from HeLa cells incubated with γ-^{32}P-ATP phosphorylate a number of polypeptides, including endogenous DNA-PK, in a DNA-dependent, salt sensitive manner (Carter et al., BBRC *157*, 535, 1988). We have begun to study the expression and regulation of this enzyme in various human cell lines using *in vitro* DNA-enhanced phosphorylation of endogenous polypeptides as an assay. Nuclear extracts from normal diploid fibroblasts contained an activity similar to HeLa cells which phosphorylated many of the same polypeptides in a DNA-dependent manner. Surprisingly, however, phosphorylation of a similar set of polypeptides was independent of added DNA in nuclear extracts from several different fibroblast lines transformed by either SV40 virus or SV40 T-Antigen alone. When purified DNA-PK was added to the transformed cell extracts, phosphorylation of the same polypeptides (including the kinase itself) was further enhanced, again without added DNA. This was not observed in extracts from HeLa cells or diploid fibroblasts. Although we can not at present rigorously exclude the possibility that small amounts of DNA might be tightly bound to proteins only in the transformed cell extracts, passage through DEAE columns did not remove the ability of these extracts to activate phosphorylation by endogenous or added DNA-PK. We are attempting to characterize the putative activator molecule and to define its relationship to expression of T-Antigen in virally-transformed cells. It is possible that disregulation of DNA-PK may accompany or contribute to the disruption of normal controls on DNA synthesis which occurs in cells transformed by SV40 and by certain other DNA tumor viruses.

H 407 **Molecular cloning of erf-1, a new member of the Ets proto-oncogene family.** Neil A. Clipstone and Gerald R.Crabtree. Department of Pathology, Howard Hughes Medical Institute, Stanford University.

The Ets proto-oncogene family represents a new class of eukaryotic transcription factors that have been strongly implicated in cellular transformation. All members of the Ets family share a short region of sequence homology with v-ets, that appears to represent a highly conserved DNA binding motif involved in sequence-specific DNA binding to purine rich target sequences. Evidence is now accumulating that members of the Ets gene family play an important role in regulatory events during normal cellular growth control. In this regard, we were interested in the potential role of Ets genes in the growth and development of human T lymphocytes. To this end, degenerate PCR primers designed to the conserved Ets DNA binding domain were used to generate a PCR probe from Jurkat cDNA, which was used to screen a Jurkat λgt11 cDNA library. In addition to the well characterized Ets family members ets-1, ets-2 and elk-1, a fourth cDNA encoding a new member of the Ets gene family was isolated, namely erf-1. This gene, which shares significant homology in its DNA binding domain with erg-2, appears most closely related to the recently described murine gene, Fli-1. This later gene has been strongly implicated in the induction of murine erythroleukemias, the role of erf-1 in human neoplasms has, however, yet to be established. The molecular cloning, characterization and expression of erf-1 will be discussed.

H 408 CREB ACTIVITY IS REQUIRED FOR GROWTH AND DIFFERENTIATION IN A THYROID FOLLICULAR CELL LINE, Woloshin, P.I., Walton, K.M., Goodman, R.H., and Cone, R.D. Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, Portland, OR 97201

The thyroid follicular cell requires elevated levels of cyclic adenosine monosphosphate (cAMP) for normal growth and expression of a differentiated phenotype. The recent discovery of cAMP regulated enhancer binding proteins (CREB) prompted us to analyze the possible role of these transcription factors in controlling thyroid cell growth and differentiated phenotype using the FRTL5 thyroid cell line as a model system. FRTL5 cells were stably transfected with an expression vector containing either the gene for wild-type CREB or a dominant negative mutant form of CREB (KCREB) which dimerizes with endogenous CREB and inactivates it.

Transfected clones were found to have a high ratio of transfected to endogenous CREB mRNA. Transient expression of somatostatin-CAT fusion gene in these clones demonstrated a greater than 50% reduction of CRE dependent transcriptional activity in the KCREB transfected clones and wild-type levels of activity in the CREB transfected clones. Parameters of growth (DNA synthesis, growth rate), and differentiation (iodide uptake, thyroglobulin mRNA levels) were then analyzed in the transfected clones.

Thymidine uptake, cell division, and iodide uptake were not affected by KCREB in the absence of elevated intracellular cAMP following a 5 day withdrawal of TSH from the medium. However, cells expressing the mutant CREB had an approximate 25% reduction in TSH stimulated thymidine uptake and growth rate, and a four-fold reduction in TSH-stimulated iodide uptake in comparison with wild-type cells or cells transfected with wild-type CREB. These experiments demonstrate that the CREB family of transcription factors is involved in both the control of growth and differentiated phenotype in the thyroid follicular cell. In contrast to iodide uptake, KREB did not significantly alter TSH stimulated thyroglobulin mRNA levels, demonstrating that while cAMP regulates most aspects of the differentiated thyroid phenotype, only some are controlled specifically by CREB.

H 409 ONCOGENE EXPRESSION IN DIFFERENTIATING PRIMORDIAL GERM CELLS, Electra C. Coucouvanis and Patricia P. Jones, Department of Biological Sciences. Stanford University, Stanford, CA 94305

Murine primordial germ cells (PGCs) undergo key developmental transitions between embryonic days 12 and 15. Motile, proliferating germ cells colonize the gonad at day 12. Proliferation ceases at day 13 in both sexes as oogonia begin to enter meiosis and spermatagonia enter mitotic arrest. The molecular mechanisms controlling these transitions are poorly understood, yet they are crucial to the production of healthy gametes. Protooncogenes, which are known to be involved in the control of growth, proliferation and differentiation in diverse cell types represent excellent candidates for genes regulating PGC developmental processes.

With the exception of *in situ* hybridization data for *c-kit* and the *c-kit* ligand, no molecular studies have focused on protooncogene expression in differentiating PGCs during the important transition period of embryonic days 12 - 15. Therefore we have examined the expression during this time frame of protooncogenes *c-kit, c-mos, c-myc, c-fos,* and *c-jun,* which have been shown to function in or be specifically expressed during various stages of gametogenesis. Due to the difficulty in obtaining sufficient quantities of PGC RNA for conventional Northern blot hybridization analysis, we have used the polymerase chain reaction (PCR) with appropriate controls to normalize the amount of cDNA in each sample and to directly compare levels of expression among male and female PGCs over a day 12 - day 15 time course for each transcript examined.

Our results, and data from others are consistent with a role for protooncogenes in mediating the differentiative processes occurring in PGCs. We find *c-myc* expressed at comparable levels in male and female PGCs at days 13, 14 and 15; however no expression is detected at day 12 (day 12 samples include PGCs of both sexes). *C-jun* shows a similar pattern of expression for days 13 -15. At day 12, expression is detected, though at significantly lower levels than the other days. *C-fos* levels in day 13 -15 PGCs of both sexes are consistently high, and we are currently examining *c-fos* expression at day 12. Increased expression of *c-myc* and *c-jun* at days 13,14, and 15 relative to day 12 supports the hypothesis that these transcripts may be involved in regulating the important transition from mitotic proliferation to the initiation of meiosis or mitotic arrest. Our preliminary findings for *c-kit* show that expression in male and female PGCs differs, at least on day 13. It is intriguing to speculate that such a difference might play some role in determining the distinct differentiative pathways taken by male and female PGCs at this stage. We are in the process of examining expression of *c-mos* during the same window of developmental time.

H 410 CONTROL OF THYROID FUNCTION, GROWTH AND DIFFERENTIATION BY MEMBRANE RECEPTORS. J.E. Dumont, C. Ledent, I. Pirson, C. Maenhaut, M. Baptist, P. Roger, F. Lamy, - Institute of Interdisciplinary Research, Free University of Brussels, B -1070 BRUSSELS.

The thyroid is controlled as other cells by several enzymatic cascades : the cyclic AMP, the phosphatidylinositol (PIP_2) and the protein tyrosine kinase (PTYRK) pathway. The intracellular signals of the first cascade is cyclic AMP, of the second diacylglycerol, inositol 1,4,5 triphosphate and calcium. The effects of these intracellular signals and of the tyrosine kinases are mostly mediated by specific cascades of protein kinases and phosphorylation. In the thyroid thyrotropin activates the cyclic AMP pathway in all species, but also the phosphatidylinositol cascade in human. PGE_1 in all species, isoproterenol in some, also activate the cAMP cascade while adenosine and α adrenergic agents inhibit it. ATP in most species, acetylcholine and α adrenergic agents in some species, activate the PiP_2 cascade. Epidermal growth factor activates the protein tyrosine kinase pathway. We have cloned several thyroid receptors: the thyrotropin receptor, adenosine A_1 and A_2 receptors. The control of thyrotropin receptor mRNA demonstrates short term upregulation, long term down regulation and an expression related to differentiation expression. However, this expression is very robust, even in thyroid cancers: - the TSH receptor is the adress of the thyroid cell which is necessary for its normal insertion in physiological regulatory network. While the PIP_2 and the PITyrK pathways induce proliferation and dedifferentiation, the TSH cyclic AMP cascade induces proliferation and differentiation. These two cells processes are therefore not incompatible. This is now demonstrated in Graves disease, in which thyroid stimulating immunoglobulins activate the cyclic AMP cascade, and in transgenic mice in which a physiologically constitutive adenosine A_2 receptor specifically expressed in thyroid, cause a generalized thyroid stimulation and the equivalent of a hyperfunctioning adenoma. The paradox of the concurrent stimulation of proliferation and differentiation may perhaps be explained by the kinetically tightly regulated expression of protooncogene cmyc.

H 411 EXPRESSION OF THE ACTIVATED HCK TYROSINE KINASE IN THE MURINE MACROPHAGE-LIKE CELL LINE BAC1.2F5 AUGMENTS MACROPHAGE ACTIVATION. B. Keith English and Angela Myracle, Department of Pediatrics, The University of Tennessee, Memphis 38103

The hck gene, a member of the src family of tyrosine kinases, is expressed in cells of the monocyte/macrophage and granulocyte lineages. In human monocyte/macrophages, hck expression increases with differentiation and is augmented by activation stimuli. We examined the potential role of hck in macrophage activation, using the murine macrophage-like cell line Bac 1.2F5 as a model. Bac1.2F5 cells require CSF-1 or GM-CSF for growth and share many characteristics of normal macrophages. Bac 1.2F5 cells expressed hck mRNA and protein, and hck expression was upregulated by CSF-1, GM-CSF and LPS. To study the role of hck in the activation of these cells, we used retroviral constructs to overexpress "activated" (Y501-F501) and "dead" (K269-E269) mutants of hck. Compared with parental cells and clones expressing the "dead" hck kinase, Bac 1.2F5 clones expressing the "activated" hck kinase accumulated more TNF mRNA (5-10x) and protein (3-4x) after stimulation with LPS. In addition, clones expressing "activated" hck accumulated appreciable amounts of TNF mRNA and protein in the absence of LPS. In parallel experiments, we used antisense oligonucleotides directed against the 5' region of hck mRNA to inhibit hck expression in Bac1.2F5 cells. Compared with untreated cells and control cells treated with sense (S) oligonucleotides, Bac 1.2F5 cells exposed to hck antisense (AS) oligonucleotides secreted 2-3 fold less TNF protein after stimulation with LPS. However, exposure of Bac 1.2F5 cells to hck antisense oligonucleotides did not affect the proliferation of these cells in response to CSF-1. These data suggest that the hck tyrosine kinase may play an important role in the signal transduction pathways involved in macrophage activation.

H 412 IDENTIFICATION OF A NOVEL SERUM-INDUCIBLE GENE IN RABBIT SMOOTH MUSCLE CELLS, Ping Feng and Gene Liau, Laboratory of Molecular Biology, American Red Cross, Rockville, MD 20855

We have used subtracted cloning procedure to isolate a cDNA (ps4) which encodes a serum inducible mRNA in vascular smooth muscle cells (SMC). DNA sequence analysis of a 1.9 Kb full length cDNA revealed one major open reading frame that can encode a 9,442 M_r protein. Comparison of the DNA as well as the putative protein sequence with various databases indicate ps4 encoded a new protein. *In vitro* translation of synthesized mRNA transcripts of ps4 revealed the presence of an approximately 15,000 M_r polypeptide. This somewhat larger apparent M_r may be due to post-translational modification. Serum stimulation of quiescent SMC in culture induced a rapid increase in the level of 1.9 Kb ps4 mRNA. Expression was detected by 1 h, peaked at approximately 4 h and became undetectable by 16 h. Induction of ps4 by serum was not abrogated by cycloheximide, indicating its expression did not require prior protein synthesis. We also assessed the ability of specific growth factors to induce ps4 mRNA level and found that both epidermal growth factor and fibroblast growth factor induced a strong and rapid increase in ps4 expression that peaked at 2 h, but insulin-like growth factor-I and surprisingly platelet-derived growth factor had little or no effect. Transforming growth factor-β was also able to strongly augment ps4 expression, however, the induction was considerably delayed, with no increase observed until 6 h and mRNA level remained high at 24 h. The analysis of ps4 mRNA level in tissues revealed a high level of ps4 mRNA in fetal muscle, esophagus, kidney and lung, but low or undetectable level in fetal aorta, heart and liver. The expression of ps4 in the corresponding adult tissues were low or undetectable. Our analysis indicate that ps4 is tightly controlled by growth factors and is potentially an important mediator of cellular proliferation and development.

H 413 REGULATION OF pp60c-src KINASE ACTIVITY DURING GROWTH STIMULATION AND IN VITRO DIFFERENTIATION OF HUMAN COLON ADENOCARCINOMA CELL LINES Gary E. Gallick, Catherine Novotny-Smith, Roy Garcia, Doris Kaneshiro,. Dept. of Tumor Biology, M.D. Anderson Cancer Center, Houston, TX 77030

The tyrosine kinase activity of $pp60^{c\text{-}src}$ is activated in nearly every human colorectal tumor, with expression and activity increasing during progressive stages of the disease. The mechanism for kinase activation is unknown, as are the potential roles of $pp60^{c\text{-}src}$ in growth regulation and differentiation of normal colonocytes. To assess these roles, we are studying changes in expression and activity of $pp60^{c\text{-}src}$ following mitogenic stimulation and induction of differentiation of model colon tumor cell lines. The kinase activity of $pp60^{c\text{-}src}$ increases following EGF, PDGF and gastrin stimulation in cell lines whose growth is stimulated by these mitogens. However, slower migrating $pp60^{c\text{-}src}$ species ($pp60^{+}$) are not detected after EGF stimulation, but are present after PDGF stimulation, sugesting that the mechanism by which $pp60^{c\text{-}src}$ activity is increased differs for these mitogens. In differentiation toward an absorptive cell phenotype, $pp60^{c\text{-}src}$ kinase and levels decrease. In contrast, such decreases do not occur in a cell line which differentiates toward a goblet cell phenotype. Growth of colon tumor cell lines with with elevated tyrosine kinase activity are sensitive to tyrosine kinase inhibitors such as herbimycin A, and tyrosine analogs such as daphnetin and fraxetin. These results suggest that regulation of $pp60^{c\text{-}src}$ play multiple roles in colonocyte growth and differentiation.

H 414 TRANSGENIC MICE EXPRESSING AN ACTIVATED ALLELE OF THE HUMAN *FPS/FES* PROTO-ONCOGENE:

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The complete human *fps/fes* locus is contained within a 13 kb genomic fragment. Transgenic mice generated with this DNA fragment display a tissue-specific pattern of human *fps/fes* expression indistinguishable from that of the endogenous mouse genes. In whole tissues highest levels of *fps/fes* expression are seen in bone marrow, spleen, thymus, lymphatic tissues and lung. Further enrichment of *fps/fes* expression is seen in macrophages and mast cells cultured from bone marrow. Transgenic mice which over-express the wild type human FPS/FES protein tyrosine kinase up to 20-fold show no overt biological phenotype.

More recently transgenic mice have been generated which carry an activating mutation in the human *fps/fes* transgene. The mutation consists of a functional myristylation consensus sequence. Transgenic mice carrying this mutant *fps/fes* allele display wide spread hyperplasia of vascular endothelial cells and signs of extramedullary hematopoiesis. *In situ* RNA analysis shows *fps/fes* expression within endothelial cells. Endothelial cell expression of *fps/fes* is not peculiar to these transgenic mice as high levels of *fps/fes* expression are seen in primary cultures of human umbilical vein endothelial cells and in a mouse hemangioma cell line. These observations demonstrate that *fps/fes* is normally expressed in both the vascular endothelial and hematopoietic lineages, and suggest a potential role of this proto-oncogene in angiogenesis and hematopoiesis.

H 415 DENSITY-DEPENDENT REGULATION OF MyoD1. Maureen A. Harrington, Raymond Daub and An Song, Indiana University School of Medicine and Walther Oncology Center, Indianapolis, IN 46202.

Expression of the myogenic determination gene, MyoD1 can be modulated by a variety of agents including growth factors and activated oncogenes. While examining the effect of interleukin-1α (IL-1α) on MyoD1 expression changes in the steady-state level of MyoD1 mRNA were observed which correlated with the cell culture density. Cultures of a myogenically determined cell line were plated either at low (0.8×10^5/10 cm^2), medium (1.6×10^5/10 cm^2) or high (3.2×10^5/10 cm^2) density in media supplemented with 10% fetal bovine serum (FBS) for 16 hours. Cultures were then switched into media supplemented with 0.2% FBS and total cellular RNA was harvested 6, 12 or 24 hours later. By 12 hours the steady-state level of MyoD1 mRNA had decreased by 60% in the low density cultures, by 40% in the medium density cultures and by 20% in the high density cultures. In all cultures, between 12 and 24 hours the steady-state level of MyoD1 mRNA began to increase. Additional experiments revealed that by 48 hours the steady-state level of MyoD1 was equivalent to that observed prior to incubation in 0.2% FBS containing media. Treatment of cultures with media containing 0.2% FBS plus IL-1α (10 ng/ml) decreased the rate of accumulation of MyoD1 mRNA between 12 and 24 hours. The density-dependent fluctuation in MyoD1 mRNA was not observed if cultures were maintained in 10% FBS. Density-dependent changes in the steady-state levels of the housekeeping gene CHO-B or the cytokine, colony stimulating factor-1 were not detected. The changes in MyoD1 mRNA levels are not due to production of factor(s) by the low density cultures which elicit the decrease in MyoD1 mRNA or by production of a factor(s) by the high density cultures which prevents the decrease. Interestingly, fibroblasts, which do not express MyoD1 can supply the needed cell-cell contact. In summary our studies suggest the degree of cell-cell contact in in vitro cultures may affect changes in MyoD1 gene expression. Whether these effects are also reflected in altered MyoD1 protein concentration is currently under investigation.

H 416 DEREGULATION OF HOX 11 IN T CELL LEUKEMIA, Masahiko Hatano, Charles W.M. Roberts, Stanley J. Korsmeyer, Howard Hughes Medical Institute, Washington University Medical School, St. Louis, MO 63110

We have cloned a novel homeobox gene, HOX 11, which is deregulated in T cell ALLs containing the t(10;14). It encodes a 330 amino acid homeobox protein that is most homologous to Hlx which is expressed in pre-B cells, and myeloid and macrophage lineages. In normal tissue, HOX 11 expression has been detected in liver but not thymus nor resting or activated T cells. Thus, redirecting HOX 11 to T cells may be leukemogenic through altered regulation of liver-specific genes or, alternatively, HOX 11 may substitute in a positive or negative fashion for a homolog normally expressed in T cells. To examine these possibilities, we made transgenic mice with HOX 11 redirected to the T cell lineage. DNA-protein and protein-protein interactions are being studied to elucidate the mechanism of HOX 11 function. In addition, we have identified a gene highly homologous to HOX 11. The homolog shares 56 of 60 amino acids in the homeodomain with HOX 11 and is identical in helix three, which has been shown to determine the specificity of the DNA binding site in other homeobox proteins. This provides the opportunity to examine interactions between HOX 11 and its homolog. HOX 11 is the first homeobox gene implicated in T cell neoplasia and constitutes a model for the aberrant expression of transcription factors in oncogenesis.

H 417 ANALYSIS OF THE N-TERMINAL PHOSPHORYLATION SITES IN MYC: POSSIBLE INVOLVEMENT OF GSK3 AND MBP KINASE

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The N-terminus of Myc has been shown to contain a potential transactivation domain (M.C.B.,10, 5914, 1990). This region is important for the transforming activity of the protein. We have found that Myc is phosphorylated *in vivo* within this region at sites that can be modified *in vitro* by GSK3. Preliminary data suggest that MBP kinase (p44^{mpk}) may also phosphorylate similar sites. These phosphorylation sites or amino acids nearby are frequently mutated in viral Mycs and in activated Mycs from transformed cell lines. Interestingly, this domain is also subjected to cell cycle-regulated modification. During M-phase, the N-terminal domain of Myc is hyper-phosphorylated by an as yet unidentified kinase. This correlates with reduced nonspecific DNA binding activity. These data suggest that several signal transduction pathways potentially merge by phosphorylating/de-phosphorylating the N-terminal domain of Myc. To characterize the biological importance of these phosphorylation sites for Myc function, they are being altered by site directed mutagenesis. We will discuss the functional analysis of these mutants.

H 418 DEVELOPMENT OF AN *IN VIVO* MODEL FOR t(6;9) ANLL, Martine Jaegle, Ton de Wit, Maarten Fornerod, Marieke von Lindern, Sjozef van Baal and Gerard C. Grosveld. Department of Cell Biology and Genetics, Erasmus University, P.O.Box 1738, 3000 DR Rotterdam, the Netherlands.

The specific (6;9)(p23;q34) chromosomal translocation is associated with a defined subtype of acute nonlymphocytic leukaemia (ANLL). The two genes located at the breakpoints on both chromosomes have been identified and characterized. The CAN gene, positioned at 9(q34), is disrupted and its 3' part is translocated to chromosome 6(p23) where it is fused in a head-to-tail fashion to the 5' part of the DEK gene. This results in the production of chimeric 5.5 kb mRNA which encodes a fusion protein of 165 kD ($p165^{DEK-CAN}$).
To investigate the direct involvement of the fusion protein $p165^{DEK-CAN}$ in the generation of ANLL, we are developing an *in vivo* model through the creation of transgenic mice. We will introduce the DEK/CAN hybrid gene under the control of either the endogenous DEK promoter or the metallothionein promoter, into mouse embryos. The results of these experiments including the characterization of the DEK promoter will be presented.

H 419 **ALTERATION OF CELL CYCLE KINETICS AND IMMUNOGLOBULIN GENE TRANSCRIPTION AS THE RESULT OF MULTIPLE AGONIST STIMULATION OF MURINE B CELLS.** Teri L. Jones and David Lafrenz, Truman Memorial VA Hospital and Department of Mol. Micro. and Immunol., University of Missouri-Columbia.

Normal murine B cells can be polyclonally activated by LPS and dextran sulfate (Dxs) which results in both proliferation (cell cycle progression) and differentiation (increased immunoglobulin gene transcription). A model of anti-idiotypic regulation of the immune response is the co-stimulation of murine B cells with LPS/Dxs and anti-immunoglobulin (α-Ig). This co-stimulation of cells results in the inhibition of both proliferation and differentiation if intact α-Ig is used but only inhibition of differentiation if $F(ab')_2$ fragments of α-Ig are used. If proliferation is assessed by acridine orange (AO) staining and flow cytometric analysis, intact α-Ig plus LPS/Dxs co-stimulated cells are observed to have entered cell cycle. This disparity between AO staining and proliferation assessed by thymidine uptake has been resolved by using BrDu-Hoechst quenching analysis to examine cell cycle kinetics.

Since both α-Ig's (intact and $F(ab')_2$ fragments) result in inhibition of differentiation and signal transduction by both α-Ig's is mediated by PIP_2 hydrolysis, we investigated the effects of Ca^{++} and PKC agonists on LPS/Dxs activated B cells. Contrary to expected results, either agonist in conjunction with LPS/Dxs resulted in an inhibition of proliferation, but not an inhibition of differentiation. Additionally, it was observed that transcription in these cells was limited to an increase in only the 2.7 kb message for membrane Ig. Finally, multiple agonist stimulation of cells and analysis of cell cycle kinetics by BrDu-Hoechst quenching demonstrated an increase in cell cycle transit time rather than a failure of initial cell activation.

The induction of c-myc and c-fos have been shown to be induced in B cells by agonists leading to cell proliferation and/or differentiation and c-jun, c-myb, and ras have been shown to be induced in other cells. The kinetics of induction and expression pattern of these protooncogenes has been assessed using a multiprobe RNA hybridization protection assay.

H 420 cREL AND NFκB COOPERATE TO REGULATE CELLULAR PROLIFERATION IN B LYMPHOCYTES, Lawrence D. Kerr and Inder M. Verma, The Salk Institute, Depart. of Mol. Biol. and Virology, San Diego, CA 92138

The product of the proto-oncogene c-*rel* and the transcription factor complex NFκB, bind to and activate transcription of genes bearing the consensus DNA-binding sequence, GGGGAATTTCC, termed κB. κB-binding factors are present ubiquitously throughout different species and cell types. The nuclear localization and DNA-binding properties of these factors are regulated by a group of inhibitors proteins, IκB (α, ß, γ). We have examined the roles of cRel and NFκB in the proliferation and differentiation of the murine B cell lines, WEHI 231 and 70Z/3. Synthetic antisense DNA oligonucleotides directed against either c-*rel* or NFκBp110 mRNA abolish the expression of the respective protein, and individually decrease the ^{3}H-thymidine incorporation of treated WEHI cells by approximately 50%. When used in combination, the antisense c-*rel* and p110 oligonucleotides inhibit the growth and proliferation of the WEHI cells by greater than 90%. No effect is observed in the presence of the appropriate sense oligonucleotides. We demonstrate that while cRel is constitutively expressed throughout the cell cycle, nuclear κB-binding activity is present only in G2. These data suggest that cRel and NFκB cooperate to regulate cellular proliferation.

H 421MOLECULAR CLONING OF fte-1, A fos-TRANSFORMATION EFFECTOR GENE WHICH ENCODES THE MAMMALIAN HOMOLOGUE OF cyc07, A PLANT GENE IMPLICATED IN CELL PROLIFERATION, Kho, C.J. and Zarbl, H., Division of Toxicology, Whitaker College of Health Sciences and Technology, Massachusetts Institute of Technology, Cambridge, MA 02139.

We have previously demonstrated that loss of the rhodamine 123 dye retention phenotype can be used to isolate revertant cell lines (non-transformed variants) from v-fos transformed cells by fluorescence-activated cell sorting. Using this approach, a revertant cell line was isolated from a population of v-fos transformed Rat-1 fibroblasts that was transfected with plasmid DNA. Southern analysis indicated that a single copy of the pMEXneo plasmid had incorporated into the genome of the revertant cell, suggesting that the transfected plasmid disrupted a transformation effector gene. We therefore performed genomic walking in the vicinity of the integration site and identified a single-copy sequence that hybridized to a unique 1.2 kb mRNA. The corresponding cDNA (fte-1) was cloned and its predicted amino acid sequence was found to be 68% identical with cyc07, a plant gene that is preferentially expressed at the G1/S boundary during the cell cycle, and 60% identical with MFT1, a yeast gene involved in protein import into mitochondria. Preliminary DNA transfection experiments indicate that the fte-1 gene does not induce transformation of Rat-1 cells, but is able to induce morphological transformation of v-fos revertant cell lines. Furthermore, transfection of Rat-1 cells with a vector that expresses the antisense fte-1 mRNA resulted in a five-fold decrease in the number of viable colonies, suggesting that fte-1 may play a role in cell proliferation.

H 422 REGULATION OF NF-κB COMPONENTS BY HUMAN CYTOMEGALOVIRUS, Timothy F. Kowalik[1], Bret A. Wing[1,2], Albert S. Baldwin[1,3,7], Jane C. Azizkhan[1,4,7], Stephen J. Haskill[1,2,5] and Eng-Shang Huang[1,2,6,7], Lineberger Comprehensive Cancer Center[1] and Departments of [2]Microbiology and Immunology, [3]Biology, [4]Pediatrics, [4]Pharmacology, [5]Obstetrics and Gynecology, and [6]Medicine, and [7]Curriculum in Genetics, School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295.

The recent identification of NF-κB as a second messenger system warrants further investigation into its regulation. Several stimuli have been shown to activate this system, among which are viruses. As NF-κB activity has been shown to play a central role in the activation of human cytomegalovirus (HCMV) immediate early genes, we investigated the effect of HCMV infection on the regulation of NF-κB genes and activity. Electrophoretic mobility shift assays (EMSA) showed the induction of several NF-κB-specific protein/DNA complexes in nuclear extracts of HCMV-infected fibroblasts. Activation of theses complexes occured within 12 hrs post infection (pi) and remained throughout the infectious cycle (72 hrs). Inclusion of bacterially expressed IκB, anti- p50, p65 or c-rel antibodies in EMSA identified the major protein/DNA complexes in these extracts as containing p50/p50 homodimers and p50/p65 heteromers. Northern analyses demonstrated the rapid induction of KBF1 (p50) RNA (4 hrs pi) as compared to the delayed increases of NF-κB and IκB transcripts (24-48 hrs pi). To determine if virus infection resulted in the inactivation and degredation of IκB, western blots of virus-infected cells were probed with anti-IκB antibodies. The results showed that protein levels of a 34 kD species (IκB-α) were not altered by infection whereas an immunologically related 42 kD protein was greatly diminished by 24 hrs pi. The disappearance of the 42 kD peptide during viral infection correlates well with the temporal activation of NF-κB CAT constructs. In addition, purified HCMV virions contain endogenous kinase activity and were tested for their ability to phosphorylate IκB in vitro. Results showed that HCMV virions phosphorylated IκB on at least two sites, similar to phosphorylation by PKC. This study demonstrates that regulation of NF-κB can occur by several mechanisms. HCMV induces NF-κB RNA levels by at least two different pathways and data are presented that suggest a novel mechanism for NF-κB activation by a virus.

H 423 SIGNALING SITE OF LAMININ WITH MITOGENIC ACTIVITY, Shunichiro Kubota, Kenichiro Tashiro, and Yoshihiko Yamada, Laboratory of Developmental Biology, NIDR, NIH, Maryland 20892

Laminin, basement membrane glycoprotein, has diverse biological activities including cell adhesion, growth, and differentiation. However, little is known concerning the signal transduction and active site involved in cell growth. In this study, we have shown that laminin and a 19-mer peptide (PA22-2), from the carboxyl terminal end of the long arm of the laminin A chain, which was previously shown to promote cell adhesion, and neurite outgrowth, stimulate thymidine incorporation and cell growth of PC12 cells. Laminin and PA22-2 were also found to induce a rapid and transient mRNA expression of c-fos and c-jun protooncogenes in PC12 cells. Further, both laminin and PA22-2 stimulate the DNA binding activity of c-Fos and c-Jun protein complex to the AP-1 site. We have also found that there is a correlation between cell growth, c-fos expression, and the ability of cell attachment to laminin or PA22-2 in different cell types. These results suggest that the PA22-2 sequence is a potent site of laminin for signal transduction and cell growth.

H 424 THE INFLUENCE OF CD28 COSTIMULATION ON THE IL-2 AND IL-4 PRODUCTION IN CD4$^+$-T-CELL CLONES, Heleen M. Kuiper, Rolien de Jong, Miranda Brouwer and Rene A.W. van Lier. Central Lab. Blood Transf. Service, Lab. of Exp. and Clin. Immunology of the Univ. of Amsterdam, Amsterdam, The Netherlands.

T cells stimulated via T-cell receptor/CD3 complex or CD2 molecules react upon costimulation of CD28 accessory molecules by an enhancement in secretion of a number of cytokines (IL-2, TNFα, IFNγ, GM-CSF, Lymphotoxin). In the present study we investigated whether differences in responsiveness to CD28 stimulation can be observed in functionally distinct types of CD4+ T-Lymphocyte clones (TLC), that produce either IFNγ (TH1-like) or IL-4 (TH2-like) in high amounts. Therefore, the effect of CD28 mAb on the production of Il-2 and Il-4 was tested in these TLC stimulated with PMA and CD2 mAb. Costimulation of CD28 appeared to increase the production of Il-2 efficiently (5-30 times), whereas the level of Il-4 was hardly influenced (1-2 times). Interestingly, two TLC that produced undetectable levels of Il-2 and Il-4 upon PMA/CD2 stimulation showed an enhanced Il-2 production and a remarkable increase of Il-4 after CD28 costimulation. With respect to these latter clones CD28 costimulation might indirectly result in an enhanced Il-4 production via the induction of a critical level of IL-2. Our data suggest that there is a differential regulation of Il-2 and Il-4 by CD28 costimulation. However Il-4 production might be indirectly increased by the action of CD28 through the induction of IL-2 production.

H 425 MUTATIONAL ACTIVATION OF Ki-*ras* and p53 BOTH OCCUR AT AN EARLY STAGE IN HUMAN PANCREATIC TUMORIGENESIS, Nicholas Lemoine & Claire Barton, ICRF Oncology Group, Hammersmith Hospital, London W12 0HS.

Highly specific point mutations of Ki-*ras* oncogene have been identified in 75% of over 200 cases of advanced pancreatic cancer. We have used PCR/SSO analysis to detect Ki-*ras* activation in micro-dissected pathology specimens and found codon 12 mutations in 83% of intraductal carcinomas (*ca in situ*). No mutations were detected in atypical hyperplasia/dysplasia, nor in intraductal papillary neoplasms which are low grade malignancies of the same ductal epithelium.

A series of 147 pancreatic cancers were examined for abnormalities of the p53 tumour suppressor gene and expression of mutant p53 was found in 60% of cases. Activating point mutations were identified in immunoreactive cases by direct sequencing and SSCP analysis. Both transition and transversion mutations were seen in conserved regions IV and V with no favoured site or base change, contrasting with the tight specificity of Ki-*ras* mutation in these tumours. Immunoreactivity for mutant p53 was observed in 60% of *ca in situ* lesions examined, implying that point mutation of p53 and Ki-*ras* both occur relatively early in pancreatic tumorigenesis.

The biological role of mutant *ras* and p53 genes in this system is being tested by retrovirally mediated gene transfer into primary pancreatic epithelial cells and antisense inhibition of gene expression.

H 426 EGF-INDUCED SIGNAL TRANSDUCTION STEPS REMAIN ACTIVE IN HEAT-STRESSED HUMAN FIBROBLASTS AND A-431 CELLS, Samuel M. Liu and Graham Carpenter, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146

A variety of changes in the function of specific plasma membrane components have been reported in cells exposed to a heat shock. We examined the consequences of heat stress on the epidermal growth factor (EGF)-induced phosphorylation of plasma membrane-associated proteins. EGF receptor tyrosine kinase activity in vitro is rapidly inactivated with heat shock. In human fibroblasts and A-431 cells, however, EGF stimulates the autophosphorylation of its receptor, even after prolonged heat stress. Phosphoaminoacid analysis of the receptor reveals that an EGF-induced increase in the levels of phosphotyrosine and phosphoserine occurs at 46 °C. In addition, EGF stimulates the phosphorylation of phospholipase Cγ at 46 °C and induces the formation of inositol phosphates to the same extent as at 37 °C. The binding and internalization of ^{125}I-labeled EGF to A-431 cells does not decrease during incubations of up to 90 minutes at 46 °C. EGF-induced dimerization of EGF receptors on the cell surface is also detected under heat shock conditions. Though EGF receptor-mediated endocytosis is not inhibited by elevated temperature, the degradation of internalized ^{125}I-EGF and/or release of EGF degradation products is greatly reduced. Our results indicate that, although the EGF receptor tyrosine kinase is rapidly inactivated at 46 °C in vitro, the EGF-mediated pathway of signal transduction through phospholipase Cγ remains intact during conditions of extreme cellular stress.

(Supported by NIH grant CA24071 and the Vanderbilt MSTP)

H 427 ACTIVATION OF COOPERATING PROTO-ONCOGENES IN *WNT-1* TRANSGENIC MICE BY MMTV INSERTION, Craig A. MacArthur*, Helen C. Kwan+, Harold E. Varmus+ and Gregory M. Shackleford*, *Division of Hematology-Oncology, Childrens Hospital and Univ. of Southern California, Los Angeles, CA 90027 and +Dept. of Microbiology, Univ. of California, San Francisco, CA 94143.

Wnt-1 transgenic mice develop sporadic mammary adenocarcinomas after a generalized hyperplasia, suggesting that *Wnt-1* contributes to, but is not sufficient for, tumorigenesis. We infected Wnt-1 transgenic mice with mouse mammary tumor virus (MMTV) to activate *Wnt-1*-cooperating proto-oncogenes. Infection decreased tumor latency from approximately 4 months to 2 1/2 months in breeding transgenic females. By Southern blotting, the majority of tumors from MMTV-infected animals possessed one or more clonal, tumor-specific proviruses. Tumors with proviruses were examined for tumor-specific expression of *int-2*, *hst*, and *Wnt-3* by northern blotting. Nearly 40% of the tumors express *int-2*, 3% express *hst* alone, and 3% express both *int-2* and *hst*. Most tumors expressing *int-2* and/or *hst* have evidence of MMTV integration near the *int-2/hst* gene region by Southern blotting. No *Wnt-3* expression or DNA rearrangement was seen. The remaining tumors with clonal proviruses show no expression of these genes. From one of these tumors, we have cloned the provirus-host junction fragment from an MMTV integration site. The cellular DNA from this clone, used as a probe, detects similar DNA rearrangements in the original tumor and at least one independent tumor. It also detects a 0.6 kb tumor-specific RNA in both tumors, in addition to a 0.9 kb RNA found in all tissues and tumors tested. Thus, this locus may represent an unexpected or novel activated proto-oncogene.

H 428 ALTERNATIVE FORMS OF MAX CAN ACT AS EITHER ENHANCERS OR SUPPRESSORS OF COTRANSFORMATION BY MYC AND RAS

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Max is a recently characterized basic/helix-loop-helix/leucine zipper protein, which forms a sequence-specific DNA-binding complex with the Myc family proteins. We present cloning of an alternatively spliced Max cDNA, which contains an additional 100 bp internal exon in the middle of the coding region. This exon introduces an in-frame translational termination codon, which leads to the formation of ΔMax, a 103 amino acid truncated form lacking 62 C-terminal amino acids of Max. ΔMax still contains the DNA-binding and dimerization motifs, but lacks both the putative regulatory domain and the nuclear translocation signal of Max. ΔMax retains the ability to form heterodimers with Myc, and binds to DNA in a sequence-specific manner, but is exclusively cytoplasmic in the absence of Myc. When tested *in vivo* in a rat embryo fibroblast Myc/Ras cotransformation assay ΔMax enhances transformation, whereas Max causes a decrease in the number of transformed foci. We suggest that the Max gene encodes both a negative (Max) and a positive (ΔMax) regulator of Myc function.

H 429 THYMIC OVEREXPRESSION OF Ttg-1, A LIM DOMAIN PROTEIN, IN TRANSGENIC MICE CAUSES T CELL ACUTE LYMPHOBLASTIC LEUKEMIA, Elizabeth A. McGuire, Catherine E. Rintoul, Gary M. Sclar, and Stanley J. Korsmeyer. Division of Hematology-Oncology, Howard Hughes Medical Institute, Washington University, St. Louis, MO 63110

We previously cloned the candidate oncogene Ttg-1 (T cell translocation gene 1) which is deregulated in t(11;14)(p15;q11) bearing T cell acute lymphoblastic leukemia. Ttg-1, normally expressed in neuronal cells, contains two novel zinc finger-like motifs termed LIM domains but lacks the homeobox domain common to other LIM transcription factors. To directly assess whether redirecting this putative transcription factor to thymus was oncogenic, we created transgenic mice expressing Ttg-1 under the control of the lymphocyte specific tyrosine kinase (lck) proximal promoter. Eight germline founders yielded transgenic mice which expressed Ttg-1 mRNA and protein in the expected tissue distribution of thymus and peripheral T cells. Aggressive, immature T cell leukemia/lymphomas has developed in all four lines which were bred. Tumors have developed in 40% of mice at risk in the highest expressing line, in 17% of mice in the second highest expressing line and 4% of mice in the two lower expressing lines. Tumors occurred at a mean age of 9 months (range 5-14.5). The mice present with large anterior mediastinal masses, circulating tumor cells and involvement of spleen and lymph nodes. These monoclonal tumors resemble human T-cell acute leukemia/lymphoma in both pathology and phenotype. The genes deregulated in T-ALL translocations (including Ttg-1, Ttg-2, scl, Lyl1, and Hox11) are all putative transcription factors usually expressed in other cell types. These results establish that the aberrant expression of Ttg-1, a neural lineage gene, is a primary oncogenic event in t(11;14)(p15;q11) bearing T-ALLs.

H 430 THE USE OF DEREGULATED MYC EXPRESSION AND DOMINANT INTERFERENCE TO STUDY NORMAL CELLULAR DIFFERENTIATION IN VIVO. Sharon D. Morgenbesser, Bhaskar Mukherjee, James Horner, Miri Bidder, Nicole Schreiber-Agus, and Ronald A. DePinho, Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461.

Myc-family genes (c-, N-, and L-myc) are believed to be regulators of normal growth and development. We have noted that changes in myc-family gene expression coincide with major developmental transitions in many cell lineages. For instance, in the murine lens we have observed that c- and L-myc transcripts are abundant in proliferating, immature cells of the anterior epithelial layer, but are undetectable in post-mitotic, differentiating cells of the equatorial region. In contrast, N-myc expression is not detectable in the immature cells, but is abundant in the differentiating lens fiber cells. Thus, coincident with the cessation of proliferation and onset of differentiation, dramatic changes in myc-family expression occur.

In order to determine the physiological significance of these changes, we have directed the late-stage expression of L-myc to the differentiating lens fiber cells in transgenic mice. In all transgenic lines generated, the late-stage expression of L-myc resulted in large nuclear congenital cataracts, disorganization of central lens fiber cells and abnormal expression of late-stage markers (MIP26, some β-crystallins). Mitotic arrest appears to be normal. These results suggest that the downregulation of L-myc is required for normal lens cell differentiation.

The non-vital role of normal lens development in the mouse also permits the generation of cell-type specific null phenotypes through the use of trans-acting dominant mutants. We have generated transgenic animals that express dominant mutants of each myc-family member in the lens. We have determined that lens-specific expression of these mutants generates significant developmental perturbations.

H 431 HUMAN B LYMPHOCYTE EARLY RESPONSE GENE EXPRESSION IN RESPONSE TO DIFFERENT PRIMARY STIMULI. John J. Murphy[1], Richard Deed[2], Joshua Newton[1], Malcolm Green[3] and John D. Norton[2], [1]Immunology Section, Division of Biomolecular Sciences, King's College London W8 7AH, [2]CRC Gene Regulation Group, Paterson Institute for Cancer Research, Christie Hospital, Manchester M20 9BX and [3]Department of Haematology, Royal Free Hospital London, NW3 2QG.

By differential screening of cDNA libraries from B chronic lymphocytic leukemia cells (B-CLL) stimulated with phorbol ester for 3 hours a panel of 12 novel early response genes (ERGs) has been isolated and partially characterized. Most of these genes are relatively B cell specific and display a highly restricted pattern of expression in response to phorbol ester in other cell types studied. The induced expression of this panel of ERGs together with other known ERGs including; c-fos, c-jun, c-myc, junD, junB, and krox 20 (EGR2) was studied in normal tonsillar B lymphocytes by Northern Blot analysis. Phorbol ester, interleukin 4 and $F(ab')_2$ anti IgM, all of which deliver primary stimuli to resting B lymphocyte populations, were used to induce ERG expression. 3 ERGs, which were previously cloned on the basis of their inducibility in B-CLL cells, were constitutively expressed at higher levels in normal B lymphocytes, were not inducible by phorbol ester, and were therefore excluded from further study. Of the remaining 15 ERGs, 14 were inducible in normal B lymphocytes by either phorbol ester or $F(ab')_2$ anti-IgM. A subset of these was also inducible by interleukin 4. Addition of interleukin 4 and $F(ab')_2$ anti-IgM together did not increase expression of any genes tested. Intriguingly, constitutive and $F(ab')_2$ anti-IgM induced expression of one ERG was inhibited by interleukin 4. These results are further evidence for different signalling pathways mediating anti-Ig and interleukin 4 stimulation of resting B lymphocytes and they highlight the complex interplay of signalling pathways at the level of gene expression.

H 432 PROLIFERATIVE ACTIVATION OF RESTING CELLS BY TWO FORMS OF FosB PROTEINS. Yusaku Nakabeppu and Mutsuo Sekiguchi, Department of Biochemistry, Faculty of Medicine, Kyushu University, Fukuoka 812, Japan

Fos and Jun transcription factors are induced during the normal course of proliferative response of resting cells to serum or growth factors. Transforming abilities of v-Fos and v-Jun proteins suggest that Fos and Jun proteins might regulate positively the normal proliferation of cells. First, we set out an experiment to address the question whether Fos protein alone can activate resting cells to proliferate. Cell lines expressing an altered form of FosB, one of the Fos family, were established by transfecting a chimeric gene composed of the hormone-binding domain of the human estrogen receptor gene and the murine fosB cDNA into rat1a cells, making it possible to test the capacity of FosB to regulate the cell proliferation. By activating the FosB chimeric protein in density-arrested or serum-starved cells with estrogen, about 50 percent of resting cells entered S phase and progressed the entire cell cycle. Previously, we discovered a new member of the Fos family, ΔFosB, which is also induced by serum and lacks the C-terminal 101 amino acids of FosB (Cell 64, 751-759, 1991). ΔFosB forms complex with Jun and binds to an AP-1 site but cannot activate transcription of an AP-1 dependent promoter nor repress the c-fos promoter. ΔFosB rather inhibits Fos/Jun transcriptional activity, suggesting that ΔFosB is a negative regulator of Jun/Fos complex. In order to evaluate the role of ΔFosB in regulation of the cell proliferation, we tested the ability of ΔFosB to activate resting cells, using the same system described above. ΔFosB could activate resting cells to enter S phase and progress the entire cell cycle while it lacks transcriptional activity. From these results we conclude that ΔFosB which inhibits Jun/Fos transcriptional activity would play an essential role(s) in regulation of cell proliferation.

H 433 ACTIVATION OF THE Ki-*ras* GENE IN LUNG TUMORS INDUCED IN THE FISCHER RAT BY THE NONGENOTOXIC CARCINOGEN BERYLLIUM. Courtney Nickell, Greg Finch, Pat Haley, and Steven Belinsky, Inhalation Toxicology Research Institute, Albuquerque, NM 87185

The activation of the *ras* family of genes has been associated with many types of human and rodent tumors. In the F344 rat, the frequency of Ki-*ras* activation in lung tumors varies depending upon the chemical carcinogen evaluated. In this study, both male and female 12 week old F344 rats received a single nose only inhalation exposure to beryllium metal at a concentration of 470 mg/m^3 for 11 minutes. This exposure resulted in mean lung burdens of 50 μg and a lung tumor incidence of 80%. Tumors were classified based on morphology as papillary adenocarcinomas. DNA from 12 lung tumors has been isolated and screened by oligonucleotide mismatch hybridization for mutations in exon 1 and exon 2 of the Ki-*ras* gene. Preliminary results reveal the presence of codon 12 GGT>GTT (8%) and GGT>TGT (33%) transversions in these tumors while GGT>GAT and GTT>AGT mutations were not detected. No mutations were present in codon 61. Mutations identified by mismatch hybridization will be confirmed by direct sequencing and additional tumors will be evaluated to further define the frequency of *ras* activation by beryllium. These observations are in contrast to the Ki-*ras* codon 12 mutational spectrum generated in the F344 rat exposed to tetranitromethane (GGT>GAT) and plutonium-239 (GGT>AGT). Results from these studies should be valuable in defining the mechanism of beryllium induced neoplasia in the F344 rat.

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H 434 HEPATIC ENDOTHELIAL CELL GROWTH- AND MIGRATION-STIMULATING FACTORS FOR LIVER-COLONIZING MOUSE LYMPHOMA CELL LINES, Garth L. Nicolson, Jun-ichi Hamada, Ori Lotan and Phillip G. Cavanaugh, Dept. of Tumor Biology, The Univ. of Texas M. D. Anderson Cancer Center, Houston, TX 77030.

Conditioned medium from mouse hepatic sinusoidal endothelial cells (HSE-CM) strongly stimulated the *in vitro* growth of highly liver-colonizing (RAW117-H10) and highly lung- and liver-colonizing (RAW117-L17) sublines compared to the poorly metastatic parental line (RAW117-P). The major growth-stimulating activity for H10 cells was not bound to DEAE Sephacel (20 mM phosphate buffer, pH 7.0). After application of the DEAE Sephacel pass-through fraction onto a Sephacryl S-200 column, the activity was eluted in the M_r ~60,000 to ~130,000 range. Analysis of this fraction by SDS-PAGE under reducing conditions demonstrated major bands of M_r ~78,000, ~51,000 and ~38,000. The M_r ~78,000 component was identified as a transferrin-like protein by Western blotting using anti-human transferrin antibody, and the activity was reduced ~60% after passage through an anti-human transferrin affinity column. HSE-CM strongly promoted the migration of liver-colonizing sublines (H10 > L17 > P). This activity was eluted at ~ 0.15 M NaCl from a DEAE Sephacel column using a linear 0-0.5 M NaCl (pH 7.0) gradient. After application of the DEAE Sephacel eluted fraction onto a Sephacryl S-200 column, the activity was eluted as a component(s) of M_r >200,000. Further purification by native gel electrophoresis resulted in a single band with activity. When analyzed by SDS-PAGE under reducing conditions, the material from the native gel migrated as two bands of M_r ~110,000 and ~67,000. Although the band of M_r ~67,000 was suggested by Western blotting analysis to be a laminin fragment, the migration of H10 cells was not stimulated by intact laminin, and the N-terminal sequence was not identified as a sequence from laminin or nidogen. The migration factor purified by native gel electrophoresis, was bound to ConA-Sepharose, but not to heparin-Sepharose or gelatin-Sepharose, and was resistant to acid (4.0) and alkaline (9.0) pH, 4 M guanidine-HCl and 6 M urea. These liver endothelial cell secreted factors may play important roles in determining the ability of tumor cells to metastasize to the liver.

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H 435 *TYROSINE KINASE GENES IN DICTYOSTELIUM,* Glen H. Nuckolls, John L. Tan, and James A. Spudich, Department of Cell Biology, Stanford University School of Medicine, Stanford, CA 94305.

The cellular slime mold *Dictyostelium discoideum* develops from a vegetative single cellular form into multicellular fruiting bodies that contain spores as well as several other differentiated cell types. Since its life cycle is relatively simple, and the organism is amenable to molecular genetic manipulation, *D. discoideum* is a good system in which to study tyrosine kinases. We have identified two *Dictyostelium* tyrosine kinase genes (DPYK1 and DPYK2) by screening an expression library with anti-phosphotyrosine antibodies. Transcription of both of these genes increases early during development. In order to determine whether the tyrosine kinases encoded by these genes are necessary for normal development of *Dictyostelium*, we have disrupted these genes using homologous recombination. Southern analysis of transformants confirmed that the kinase genes were altered such that their expression should yield a truncated protein with no kinase activity. Cell lines in which DPYK2 has been disrupted were able to form fruiting bodies that appeared normal and generated viable spores. We are analyzing other aspects of development of these cells and other cells in which DPYK1 has been disrupted.

H 436 ONCOGENIC ACTIVATION OF TRK PROTO-ONCOGENE (A NERVE GROWTH FACTOR RECEPTOR) IN HUMAN PAPILLARY THYROID CARCINOMA - Marco A. Pierotti, Italia Bongarzone, Angela Greco, Monica Miozzo, Gabriella Sozzi, Paolo Radice and Giuseppe Della Porta - Division of Experimental Oncology A. Istituto Nazionale Tumori, Via G. Venezian 1, 20133 Milan, Italy.

By transfection assay on NIH3T3 of DNA from 40 human papillary thyroid carcinomas, we have identified 7 positive cases showing an activation of TRK proto-oncogene. The latter has been recently identified as a nerve growth factor receptor and designated as NTKR1. The molecular characterization of its transforming rearrangements has revealed that NTKR1 tyrosine kinase domain can be fused with 5' foreign sequences provided by different genes. In three cases the 5' end activating sequences derived from a gene coding for an isoform of non-muscle tropomyosin (TPM3) as in the case of the original TRK oncogene described by Martin-Zanca et al. in M. Barbacid laboratory (Nature 319, 743-748, 1986). In other three cases tropomyosin sequences were replaced by portions of the 5'end of TPR, a gene originally identified as the activating sequence of the MET proto-oncogene. The TRK-activating sequence of the last tumor is currently under investigation and we have already determined that it does not belong neither to TPM3 nor to TPR genes. We have mapped NTKR1 to chromosome 1q32q41 and TPM to the same chromosomal region (1q31). TPR has been also reported to map on chromosome 1q; thus it can be derived that intrachromosomal rearrangements provide the mechanism for the proto TRK oncogenic activation. Finally, the biochemical characteristics of the different NH2 terminus peptides of the TRK-transforming sequences will be compared to identify the mechanisms of activation of the tyrosine kinase activity associated to the transforming capability of the fusion products.

H 437 ROLE OF RAS IN ADIPOCYTIC DIFFERENTIATION OF 3T3 L1 CELLS. Almudena Porras, Manuel Benito, Angel R. Nebreda and Eugenio Santos. Laboratory of Cellular and Molecular Biology, National Cancer Institute, NIH, Bethesda, Maryland 20892

Mammalian 3T3L1 cells differentiate into adipocytes after continuous exposure to pharmacological doses of insulin or physiological doses of insulin-like growth factor I (IGF-I). Expression of transfected *ras* oncogenes induces terminal adipocytic differentiation of these cells in the absence of externally added insulin or IGF-I. In contrast, 3T3 L1 cells transfected with normal *ras* genes or other tyrosine kinase oncogenes did not undergo differentiation into adipocytes.

Using standard differentiation protocols including insulin treatment, more than 90% of untransfected 3T3 L1 cells are induced to differentiate to adipocytes. However, only about 40% of cells transfected with the dominant inhibitory *ras* mutant (H-*ras*$^{lys12, ser186}$) differentiated, suggesting a competition of the transfected mutant with endogenous ras. Exposure of untransfected 3T3 L1 cells to insulin stimulated the formation of the active p21ras.GTP complex and resulted in almost two fold increase of the p21ras.GTP/ p21ras.GDP ratio, confirming the participation of the endogenous ras in insulin signaling in these cells. Interestingly, while PDGF stimulation caused marked tyrosine phosphorylation of GAP in these cells, insulin stimulation did not.

These results indicate that ras proteins participate in insulin signaling pathways and suggest an alternative mechanism of ras activation in these cells, involving other regulatory elements besides GAP.

H 438 EGF, bFGF and NGF INDUCE MODIFICATION OF PHOSPHATIDYLINOSITOL 3-KINASE ACTIVITY in PC12 CELLS, Simona Raffioni and Ralph A. Bradshaw, Department of Biological Chemistry, University of California, Irvine, CA 92717

Stimulation of PC12 cells with EGF, bFGF and NGF results in rapid increases in intracellular protein tyrosine phosphorylation. However few substrates for the induced protein tyrosine kinases have been identified. Using anti-phosphotyrosine (anti-P-Tyr) antibodies to immunoprecipitate phosphotyrosine containing proteins, we have observed increase levels of phosphatidylinositol (PI) 3-kinase activity in cells stimulated with these factors. After 2 minutes of stimulation EGF induces fifteen- to twentyfold, bFGF two- to threefold, and NGF eight- to tenfold increases in the level of PI 3-kinase activity at concentration of EGF, bFGF and NGF in the range required for stimulation of either mitogenic or neurotrophic response in PC12 cells. However there is no apparent stimulation of PI 4-kinase which produces the 4,5 isomer of phosphatidylinositol bisphosphate. The greater stimulation by EGF, which induces only a mitogenic response in these cells, suggests that this enzyme, and therefore the production of the 3,4 isomer, is not required for the neurotrophic activities of NGF and bFGF. It is consistent with their observed behaviour of inducing an initial mitogenic response, prior to the onset of neurite production. Supported by NS (19964), the American Cancer Society (BE44) and the AMGEN Corp.

H 439 MURINE HCK IN UNDIFFERENTIATED EMBRYONAL STEM CELLS: EXPRESSION DECLINES FOLLOWING DIFFERENTIATION, Steve Ralph, Matthias Ernst, Juliana Chang, Lindsay Williams, Peter Lock, Edouard Stanley and Ashley Dunn, Ludwig Institute for Cancer Research, PO Royal Melbourne Hospital, Melbourne, Australia, 3050.

The *src* related tyrosine kinase, *hck*, is expressed predominantly in mature cells of the myeloid and B-lymphoid lineage. In the mouse, two isoforms, $p59^{hck}$ and $p56^{hck}$ have been shown to be generated by utilisation of alternative translational initiation codons. The same alternative start codons are present in the human cDNA allowing for the production of two isoforms of human *hck*. While both murine *hck* isoforms are myristylated and found in the cell membrane, significant levels of non-myristylated $p59^{hck}$ are present in the cytoplasm of various cell lines examined (1). We have recently extended these studies to show that *hck* is expressed in embryonal stem (ES) cells. Moreover, the pattern of differential sub-cellular localisation with both *hck* isoforms in the plasma membrane and $p59^{hck}$ in the cytosol is also present in ES cells. The expression of both *hck* isoforms has been shown to decline when ES cells are induced to differentiate by withdrawl of LIF from the culture medium.

To determine whether expression of *hck* is intimately associated with the differentiated state of the cell we have established ES cell lines expressing an up-mutant of *hck* (499Y-F) under the control of the PGK promoter. Upon withdrawl of LIF the cells differentiate despite maintaining high levels of *hck* kinase suggesting that *hck* kinase reflects the state of cellular differentiation rather than by playing a direct role in its regulation.

1) Lock, P. et al (1991), Mol Cell Biol 11,4363-4370.

H 440 CHARACTERIZATION OF A GROWTH-REGULATED CA^{2+} TRANSPORT SYSTEM IN *SACCHAROMYCES CEREVISIAE*, Philip M. Rosoff and Rajani Kanteti, Depts. of Pediatrics and Physiology, Tufts University School of Medicine & The New England Medical Center, Boston, MA 02111.

An influx of extracellular calcium is associated with the stimulation of a large number of mitogenic signaling receptors in mammalian cells. We have previously characterized this system in T lymphocytes as a membrane potential-sensitive Ca^{2+} transporter that can be inhibited by the stilbene disulfonate, DIDS. However, attempts to isolate the channel protein have proven frustrating in these cells due to its low abundance and the lack of high affinity probes. It has recently been shown by others that exponentially growing haploid cells of the budding yeast, *Saccharomyces cerevisiae*, have an influx of Ca^{2+} when treated with the α mating factor. Prevention of this Ca^{2+} influx results in an aberrant mating response. This suggests that it may subserve a signaling function in this setting, since log-phase haploid cells can grow quite well in the total absence of extracellular Ca^{2+} for at least 7-8 generations. We were interested to see if this system was homologous to that found in T cells. We have studied three different yeast phenotypes (wild type + α, and cdc7 and cdc28 mutations) to show that the Ca^{2+} influx is associated with the cessation of growth in these cells. This is the opposite of what is found in mammalian cells in which an increase in $[Ca^{2+}]_i$ accompanies entry into the cell cycle. Like that in T cells, the yeast Ca^{2+} influx is inhibited by low concentrations of La^{3+}, Gd^{3+}, Ni^{2+}, and Mn^{2+}, and DIDS, but not by the dihydropyridine class of Ca^{2+} channel blockers. It also appears to be sensitive to changes in membrane potential since depolarization inhibits it; however, in contrast to T cells, hyperpolarizing agents such as valinomycin have no effect. Likewise, uncouplers such as CCCP and DNP are also ineffective. These data demonstrate that *S. cerevisiae* has a growth-regulated Ca^{2+} transport system that is homologous to that found in higher eukaryotes. We are taking advantage of these observations to devise strategies for generating mutant cells in order to isolate the Ca^{2+} channel gene. This work has been supported by grants from the American Cancer Society and the Leukemia Society of America.

H 441 ANALYSIS OF ADENYLATE CYCLASE AND A PUTATIVE REGULATORY PROTEIN ENCODED IN THE AFRICAN TRYPANOSOME VARIABLE SURFACE GLYCOPROTEIN EXPRESSION SITE, Douglas T. Ross and Harvey Eisen, Fred Hutchinson Cancer Research Center and the Dep't of Pathology, University of Washington School of Medicine, Seattle WA.

African trypanosomes are protozoan parasites that replicate in the bloodstream of infected mammals and evade the host immune response by varying their dense antigenic coat termed the Variable Surface Glycoprotein (VSG). The VSGs are expressed from telomere linked expression sites that also encode a number of additional ORFs termed Expression Site Associated Genes (ESAGs). We have shown that a partial ORF encoded on a T. equiperdum expression site clone encodes a protein that is homologous to the catalytic domain of yeast adenylate cyclase, and has adenylate cyclase activity when expressed in a yeast strain deleted for adenylate cyclase (Ross, D.T. et al. EMBO J., 10:2047, 1991). The cyclase is a member of a gene family of greater than 10 members. A full length cDNA clone of a member of the gene family encodes a predicted protein with a single carboxy terminal adenylate cyclase catalytic domain, flanked upstream by a single transmembrane region, and a large putative extracellular domain. Located adjacent to the cyclase ORF in the expression site is a second ORF with a leucine-rich repeat sequence motif similar to a regulatory domain of yeast adenylate cyclase and a number of other proteins implicated in protein-protein interactions. We are currently investigating whether trypanosome adenylate cyclase is activated by dimerization, and whether the protein containing leucine rich repeats modulates adenylate cyclase activity when co-expressed with adenylate cyclase in yeast.

H 442 NOVEL HUMAN ROLIPRAM-SENSITIVE PHOSPHODIESTERASE (PDE): cDNA CLONING, EXPRESSION AND HOMOLOGY TO RAT FAMILY IV PDEs, Earl R. Shelton, Rena Obernolte, Sunil Bhakta, Robert Alvarez, Vatche Kalfayan and Kurt Jarnagin, Syntex Research, Institute of Bio-organic Chemistry, Palo Alto, CA 94304

Phosphodiesterases (PDEs) can be classified into five families on the basis of their substrate specificity and profile of inhibitors and stimulators. Family IV PDEs (F-IV) are characterized by their low K_m and specificity for cAMP as well as inhibition by rolipram and related drugs. In the rat the F-IV PDEs comprise four distinct genes: A, B, C and D. Using the 43D human lymphocytic cell line we have isolated a 3.8 Kb cDNA by low stringency screening with a rat F-IV gene B PDE probe. Expression of the human cDNA in E. coli results in novel cAMP-specific PDE activity which is rolipram sensitive. An open reading frame of 1695 bp predicts a 565 amino acid protein with near identity to the rat F-IV **gene B** PDE; at the nucleotide level the homology is 84%. In contrast, our human F-IV gene B PDE has distinctive differences from a related cDNA clone isolated by others [Livi, et al. (1990) Mol. Cell. Biol. 10, 2678-2686] from human monocytes. Our analysis places the monocyte clone in the **gene A** category of F-IV PDEs.

Use of short probes specific for the human gene A and B cDNAs to analyze genomic southern blots demonstrates that human F-IV PDE genes A and B are distinct. A northern analysis of mRNAs from fractionated normal human leukocytes using the short probes shows gene specific messages of differing size and relative abundance. Transcripts of gene A (3.0 and 4.6 Kb) appear to be generally less abundant than gene B transcripts (3.7 and 4.3 Kb). Analysis of the gene B cDNA clones described herein suggested that alternative 5'-end DNA sequences are used. This was confirmed by PCR analysis of poly-A^+ mRNA from fractionated human leukocytes.

Finally, the distribution of F-IV mRNAs in rat tissues was examined using short probes specific for rat genes A, B, C and D. The results show a distinct transcript pattern for each probe with a wide-spread distribution among tissues. Nonetheless, abundant expression was observed only in a subset of tissues, which differed among the four genes. These results demonstrate a phenomenal complexity in the genetic expression of F-IV PDEs in rats and humans.

H 443 POSITIVE REGULATION OF PANCREATIC BETA-CELL GROWTH, Åke Sjöholm, Department of Endocrinology, Karolinska Institute, Karolinska Hospital, Box 60500, S-10401 Stockholm, Sweden.

A common denominator of all forms of diabetes mellitus is insufficient extent of pancreatic beta-cell replication needed to expand the beta-cell mass in order to compensate for the decreased insulin output by the pancreas resulting in hyperglycemia. A limited number of substances (e.g., glucose, GH, amino acids and cAMP) have been identified as stimulators of beta-cell proliferation in vitro. However, little is known about the intracellular pathways involved in transducing the mitogenic signals of these factors into a proliferative response. I have found that glucose, GH, the protein kinase C activating phorbol ester TPA, lithium, amino acids and PDGF+IGF-I caused stimulation of fetal rat beta-cells in tissue culture along with an increase in their polyamine content, while EGF, TGF-alpha or PDGF alone did not affect these parameters. However, when the increased polyamine content was blocked by use of enzymatic inhibitors the increased proliferative rate remained or in some cases were even amplified, suggesting that polyamines do not mediate the mitogenic signals of these factors. Glucose also raised the beta-cell cAMP content, and the stimulatory cAMP analog Sp-cAMP[S] enhanced beta-cell proliferation. However, the cAMP antagonist Rp-cAMP[S] failed to prevent the mitogenicity of glucose (and GH, TPA and lithium), suggesting that the increased cAMP content is not necessary for the growth promoting effect of the sugar. Down-regulation of protein kinase C by prolonged exposure to high concentrations of TPA prior to addition of growth factors also failed to influence their mitogenic action, suggesting that protein kinase C activation is not mandatory in this context. The guanylyl cyclase activator ANP, known to elevate beta-cell cGMP levels, or the stimulatory and membrane permeant analog 8-PCPT-cGMP also did not affect beta-cell growth, thus ruling out a requirement for cGMP in this process. However, the mitogenicity of glucose, GH, lithium and TPA (but not forskolin) was attenuated by pertussis toxin pretreatment, indicating a possible involvement of GTP-binding proteins in transduction of the mitogenic messages by these growth factors.

H 444 EFFECT OF TRANSFECTION OF 2B4 T-HELPER HYBRIDOMA WITH cAMP BINDING SITE-MUTATED REGULATORY (R1m) SUBUNIT OF PK-A ON T-CELL RECEPTOR TRIGGERED PROGRAMMED CELL DEATH, Hirotaka Sugiyama, Melissa Hunter and Michail Sitkovsky, LI, NIAID, NIH, Bethesda, MD 20892

Different promoters were utilized to construct expression vectors used in transfection of 2B4 T-helper cells with mutated R subunit (R_m) of the cAMP-dependent protein kinase (PK-A). It was expected, that transfected R_m subunit will bind to and irreversibly inactivate the catalytic C subunit and will therefore interrupt the propagation of transmembrane signalling in lymphocytes which may be mediated by the increases in the cAMP. The goal was to evaluate the effect of the partial inactivation of the PK-A on such TCR-triggered responses as IL-2 gene transcription, expression and secretion and on TCR-triggered T-helper cell death. The inhibitory effect of transfected R_m on the PK-A activity was confirmed using synthetic peptide phosphorylation assay with cell extracts. It is shown that R_m transfectants are much more resistant to the TCR-triggered death than control untransfected and neo-transfected cells, implicating PK-A in death pathways. These transfectants were also much less efficient in production of IL-2 in response to activating anti-TCR mAb. It is concluded that PK-A participates in important biochemical pathways, that are triggered by the TCR occupation by the antigen. It is suggested that PK-A plays similar role in TCR-triggered responses in T-cells and in sIg-triggered responses in B-cells, since transfection of B-lymphoma cells by R_m also resulted in their protection from anti-Ig-induced death and in enhanced reversibility of anti-Ig effects by LPS. The described transfectants promise to provide a convenient experimental system to study biochemical pathways involved in programmed cell death in lymphocytes.

H 445 RAPID INDUCTION OF NF-KB AND REL INHIBITORY PROTEIN, RL/IF-1, AND NF-KB SITE BINDING PROTEIN, PHF-1, IN REGENERATING LIVER R. Taub, M. Tewari, P. Dobrzanski, J-C Hsu, K. L. Mohn, and R. Bravo Department of Human Genetics, HHMI University of Penn School of Medicine, Phila., PA 19104 and Molecular Biology Dept, Bristol Myers Squibb Pharmaceutical Research Institute, Princeton, N.J. 08543-4000.

During the early growth response of regenerating liver and mitogen-treated cells mRNAs encoding several members of the NF-KappaB (NF-KB) and rel family of transcription factors are induced. In addition, we have found that a novel highly induced immediate-early gene in regenerating liver encodes a RL/IF-1 (regenerating liver inhibitory factor), a protein with cdc-10 type repeats. Proteins with these repeats have varying activities ranging from transcription factors to membrane associated proteins. RL/IF-1 is most homologous to the cleaved portion of p105 NF-KB and BCL-3, and less homologous to ankyrin and notch. Using mobility shift assays, we have demonstrated that RL/IF-1 is similar in activity to I-KappaB proteins in that it inhibits the binding of p50/p65 NF-KappaB and rel family proteins to NF-KB sites. It is intriguing that both NF-KB/rel family proteins and RL/IF-1, an IKB-like inhibitory protein, are induced simultaneously during hepatic regeneration. Using mobility shift assays, we examined changes in NF-KB binding activity during liver regeneration, and discovered a rapidly induced novel NF-KB site binding factor designated PHF-1 (posthepatectomy factor). PHF-1 is induced within minutes posthepatectomy in a protein-synthesis independent manner with peak activity at 30-90 minutes, and is not induced by sham operation. The PHF-1 complex migrates near the p50/p50 homodimeric NF-KB protein, but does not interact strongly with anti-p50 or other rel-specific antibodies. Because the PHF-1 complex is induced immediately posthepatectomy in the absence of new protein synthesis, PHF-1 could have a role in the induction of liver-specific immediate early gene expression in regenerating liver.

H 446 THE ROLE OF TYROSINE KINASE ACTIVATION IN REGULATING PC12 CELL DIFFERENTIATION, Monica L. Vetter, J. Michael Bishop, G.W. Hooper Foundation, University of California, San Francisco, CA 94143-0552

A number of peptide hormones have been identified that promote survival and differentiation of neuronal cells. An important model system for studying the effects of some of these neurotrophic factors is the rat PC12 pheochromocytoma cell line. PC12 cells resemble adrenal medullary chromaffin cells and survive in culture in the absence of nerve growth factor (NGF). When exposed to NGF, PC12 cells differentiate towards a sympathetic neuron-like phenotype: they extend neurites, they become electrically excitable due to the expression of voltage-dependent sodium channels, and they express a large number of neuronal-specific genes.
Two receptors have been identified that interact with NGF. A cDNA clone encoding a 75-80 kd receptor (p75) was isolated that binds NGF with low affinity (K_d of 10^{-9} M) when expressed in a fibroblast cell line. Recently, the product of the proto-oncogene trk ($p140^{trk}$) was also shown to directly bind NGF. It has remained controversial whether p140trk alone is capable of binding NGF with high affinity (K_d of 10^{-11} M) and mediating the NGF signal or whether it must cooperate with p75 to form a functional high affinity receptor complex.
In order to address this issue we have generated chimeric receptors consisting of the extracellular and transmembrane domains of the platelet-derived growth factor (PDGF) receptor fused to the intracellular domain of $p140^{trk}$ or p75. Since the PDGF receptor is not expressed in PC12 cells, when introduced into these cells the activity of the chimeric receptors can be controlled by PDGF application without activation of endogenous receptors. Using these chimeric receptors the following questions will be addressed: When introduced into PC12 cells does the PDGF receptor-$p140^{trk}$ chimera promote differentiation upon treatment with PDGF? If not, will co-expression of the PDGF receptor-p75 chimera produce a differentiation response? Finally, does the PDGF receptor-trk chimera alone have any signalling properties in PC12 cells?

H 447 C-JUN EXPRESSION REGULATED BY DISTINCT SIGNAL TRANSDUCTION MECHANISMS IN GROWTH-ARRESTED SV40-TRANSFORMED CELLS DURING INSULIN- AND VANADATE-INDUCED MITOGENESIS, Hanlin Wang and Robert E. Scott, Department of Pathology, The University of Tennessee Medical Center, Memphis, TN 38163
Insulin- and vanadate-induced mitogenesis are both associated with the induction of the immediate early gene c-jun in SV40 large T antigen-transformed 3T3 T cells designated CSV3-1. (Wang and Scott: J. Cell. Physiol. 147:102-110, 1991; Wang et al: Cell Growth & Differ. in press). To further elucidate the early signal transduction pathways employed by insulin and vanadate, mechanisms to regulate c-jun by insulin and vanadate have now been investigated. In growth-arrested CSV3-1 cells, down-regulation of protein kinase C (PKC) by prolonged exposure to TPA or by PKC-specific inhibitor staurosporine does not affect c-jun induction by insulin and vanadate, suggesting that both insulin and vanadate act in a PKC-independent manner. Insulin's effect on c-jun induction can be inhibited by pertussis toxin (PT) which effects G protein but not by genistein, a specific tyrosine kinase inhibitor. In contrast, vanadate's effect on c-jun can be inhibited by genistein but not by PT. These data suggest that a PT-sensitive G protein is involved in the insulin signal transduction system whereas tyrosine kinase activation is involved in the vanadate signal transduction system. This difference is supported by the findings that insulin induces the non-tyrosine phosphorylation of a 35 kDa cellular protein whereas vanadate does not. In addition, insulin induces the tyrosine phosphorylation of the insulin receptor beta-subunit whereas vanadate does not. Additional differences in the effects of insulin and vanadate also exist. Depletion of polyamines, particularly spermidine, by difluoromethylornithine treatment results in a dramatic decrease in c-jun RNA levels induced by insulin but shows no effect on c-jun induction by vanadate. Additional data suggest that cAMP-dependent and Ca^{2+}-dependent pathways may not be of major importance in insulin- or vanadate-induced c-jun expression. These observations together indicate that the c-jun induction by insulin and vanadate in CSV3-1 cells is mediated by distinct PKC-independent signal transduction mechanisms.

H 448 T-CELL-SPECIFIC EXPRESSION OF V-MYB IN TRANSGENIC MICE, Kathy Weston, Pratipa Badiani, [1]Andrew Edwards and [2]Dimitris Kioussis, Institute of Cancer Research, Chester Beatty Laboratories, London SW3 6JB, [1]Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, and [2]National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA
V-myb , and its cellular progenitor c-myb, are DNA-binding transcription activators implicated in the control of hemopoietic differentiation; high levels of either v- or c-myb protein inhibit differentiation, whilst c-myb expression appears necessary for cell growth. C-myb protein is detected most abundantly in immature T cells, and also during T cell activation, but almost nothing is known about the effects of c-myb on T cell ontogeny. To examine possible roles for v- and c-myb in T cell development, we have established two transgenic mouse lines in which the v-myb protein is specifically expressed at high levels in T cells. Unlike endogenous c-myb, the transgene is not downregulated during thymocyte maturation, and might therefore be expected to perturb normal development. The effects of the transgene on T cell growth and differentiation are currently being determined.

H 449 STIMULATION OF PROLIFERATION AND SUPPRESSION OF DIFFERENTIATION IN HUMAN KERATINOCYTES INFECTED WITH RECOMBINANT RETROVIRUSES ENCODING HUMAN PAPILLOMAVIRUS TYPE 18 E6 AND E7 GENES, Craig D. Woodworth[1], Shinta Cheng[2], Scott Simpson[1], Louise T. Chow[2], Thomas R. Broker[2], and Joseph A. DiPaolo[1]. Laboratory of Biology, NCI, Bethesda, MD 20892[1], and Dept. of Biochemistry, Univ. of Rochester School of Medicine, Rochester, NY 14642[2].
A subset of human papillomavirus (HPV) DNAs are detected in most genital dysplasias and cancers suggesting that these viruses perturb epithelial growth and differentiation. The E6 and E7 genes of HPV-18 induce immortality in cultured genital keratinocytes and the immortal cell lines display aberrant squamous differentiation in vitro. To examine whether the E6 and E7 proteins directly alter keratinocyte growth and differentiation high-titer recombinant retroviruses were constructed for efficient transfer and expression of HPV-18 early genes (E6, E6*, and E7) in cultures of normal human keratinocytes. Infection with retroviruses encoding the E6 and E7 stimulated cell proliferation, reduced the requirement for bovine pituitary extract, and induced immortality. E6 and E7 also delayed but did not prevent the onset of terminal squamous differentiation. However, E7 expression did not alter the responsiveness of keratinocytes to growth inhibition by TGFβ1. The magnitude of effects on growth differentiation of cultured cells was directly related to levels of E7 protein expression. Thus, the primary effect of HPV-18 E6 and E7 gene expression is to stimulate proliferation and delay differentiation of keratinocytes.

H 450 LECTIN INDUCED DIFFERENTIATION OF MOUSE AND HUMAN LEUKEMIA CELLS, Mayumi Yagi, Elenita Yong, Marilyn Parsons, Brad Greenfield, and Katherine Gollahon, Seattle Biomedical Research Institute, Seattle, WA 98109.
Lectins, carbohydrate-binding proteins often derived from plant seeds, have long been used to stimulate proliferation and differentiation of mature lymphocytes. The effects of lectins on other hematopoietic cells and on immature cells is less well-characterized. We have studied the effects of Jacalin, a lectin derived from jackfruit (*Artocarpus heterophylla*, formerly *A. integrifolia*) seeds, on the mouse pre-B cell line 70Z/3 and the human leukemia line K562. Previous studies indicate that the addition of Jacalin to mouse long-term bone marrow cultures results in an increase in the number of surface IgM-positive B cells in the cultures. The pre-B cell line 70Z/3 responds to bacterial lipopolysaccharide by translocating the transcriptional factor NF-κB to the nucleus and expressing increased levels of surface IgM. However, in contrast to bone marrow cells, 70Z/3 cells do not express increased levels of surface IgM in response to Jacalin. Jacalin does not induce translocation of NF-κB to the nucleus, and in fact appears to interfere with LPS-induced translocation of this transcriptional factor. Culture of K562 cells with Jacalin results in dramatic and rapid morphological alterations. Within minutes, the cells become adherent and spread on the tissue culture surface. Prolonged culture in the presence of Jacalin results in the deposition of extracellular material containing fibronectin and several other cell-derived polypeptides. In addition, the Jacalin-treated K562 cells express increased levels of cell surface CD61 (integrin β3) and CD14, a monocyte marker. Analysis of the phosphotyrosine content of Jacalin-treated K562 cells reveals that an increase in the tyrosine phosphorylation of two proteins is observed 24 hours after the addition of Jacalin. Although the kinetics of the phosphotyrosine alterations indicate that this is a late event in Jacalin-mediated phenotypic changes, these alterations may be important in maintenance of the altered phenotype. The results of these analyses indicate that the lectin Jacalin has a variety of effects on immature hematopoietic cells, and may be capable of inducing differentiation to more mature phenotypes.

H 451 N-myc INDUCES Ig/c-myc TRANSLOCATION-FREE PLASMACYTOMAS IN N-myc TRANSGENIC MICE, Yisong Wang, Hiroyuki Sugiyama, Håkan Axelson, Chinmay Panda, Magdalena Babonits, Averil Ma*, Frederic W. Alt*, George Klein and Francis Wiener, Dept. of Tumor Biology, Karolinska Institute, Box 60400, S-10401, Stockholm, Sweden; *The Howard Hughes Medical Institute and Dept. of Biochemistry and Microbiology, Columbia Univ., College of Physicians and Surgeons, New York, NY 10032
Mouse plasmacytomas(PCs) induced by pristane oil, with and without A-MuLV, regularly carry one of three alternative chromosomal translocations that juxtapose c-myc to immunoglobulin heavy or light chain loci respectively. In order to test another member of the myc family, N-myc, could replace Ig/c-myc translocation in PC-genesis, we have treated Emu-N-myc transgenic mice with pristane and helper free A-MuLV. Out of 20 mice that received single pristane injection followed by A-MuLV infection, 17(85%) developed PCs with a mean latent period of 54±20 days. In a corresponding group that only received a single pristane injection, 5(83%) out of 6 transgenics developed PCs with a mean latency of 142±32 days. All transgenics that received three monthly injections of pristane, developed PCs (15/15) with a mean latency of 128±20 days after the first pristane injection. All PCs derived from pristane+ A-MuLV treated N-myc transgenic mice expressed both the N-myc transgene and v-abl. None of the PCs that have appeared in N-myc transgenics, whether treated with pristane alone or pristane +A-MuLV, expressed c-myc and none of the tumors carried the usual PC-associated translocations. We thus provide evidence that N-myc, when activated, has the same capacity as the deregulated c-myc for the plasma cell transformation.

H 452 MOLECULAR MIMICRY OF THE ACTION OF INSULIN IN ONCOGENE EXPRESSION AND TRANSCRIPTION FACTORS BY 3-DEAZAADENOSINE DURING THE INDUCTION OF 3T3-L1 FIBROBLASTS TO ADIPOCYTES, G. C. Zeng, J. R. Dave, S. A. Brugh, J. S. Estrada, and P. K. Chiang, Applied Biochemistry Branch, Walter Reed Army Institute of Research, Washington, DC 20307-5100.
3-Deaza-adenosine (3-deaza-Ado) is an indirect inhibitor of methylation by functioning as inhibitor and also as alternative substrate of S-adenosylhomocysteine (AdoHcy) hydrolase. In cells treated with 3-deaza-Ado, AdoHcy accumulates, generally leading to an inhibition of methylation reactions. Confluent 3T3-L1 fibroblasts treated with either 3-deaza-Ado or insulin differentiate into fat cells [Science 211, 1164 (1981)]. Both 3-deaza-Ado and insulin produced in confluent 3T3-L1 cells a rapid but transient expression of mRNA for proto-oncogenes c-*fos* and c-*jun* within 30-60 min, after which the mRNA of both proto-oncogenes became undetectable. In comparison c-*myc* proto-oncogene became detectable after 2 h, and then declined after 4 h. Electrophoretic mobility shift assays showed an increase in the levels of transcription factors Ap1, Ap2 as early as 1 h and was still evident up to 6 h following treatment with either 3-deaza-Ado or insulin. In contrast, no noticeable difference was observed in the level of the transcription factor Sp1. In cells transfected with antisense *c-fos*, neither 3-deaza-Ado nor insulin could effect a differentiation response. Thus 3-deaza-Ado mimics the action of insulin most likely by inhibiting key early methylation reaction(s), leading to the transient expression of c-*fos* and c-*jun* proto-oncogenes and the attendant transcription factors Ap1 and Ap2 during the initial phase in the differentiation of the 3T3-L1 fibroblasts to adipocytes.

Transcription and Gene Expression

H 500 ACTIVATION OF THE EGR-1 PROMOTER BY V-FPS IS DEPENDENT UPON c-HaRAS AND c-RAF-1

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The oncogenic protein-tyrosine kinase v-Fps induces expression of the transcription factor Egr-1 via a protein kinase C (PKC)-independent intracellular signaling mechanism. v-HaRas and v-Raf, the constitutively active forms of c-HaRas and c-Raf-1 respectively, also activate the Egr-1 promoter in transient expression assays. To determine if the ability to activate the Egr-1 promoter by v-Fps, v-HaRas and v-Raf was due to a dependence upon their normal cellular counterparts in an intracellular signaling pathway initiated by v-Fps, we employed dominant inhibitory mutants of c-HaRas and c-Raf-1 to inhibit intracellular signals leading to the activation of the Egr-1 promoter. Activation of the Egr-1 promoter by v-Fps was inhibited by both c-HaRas and c-Raf-1 inhibitory mutants. Activation of the Egr-1 promoter by v-HaRas was blocked by the inhibitory c-Raf-1 mutant; however, activation of the Egr-1 promoter by v-Raf was unaffected by the inhibitory c-HaRas mutant. These data suggest that the PKC-independent intracellular signaling mechanism used by v-Fps to induce Egr-1 expression involves both c-HaRas and c-Raf-1, and that c-Raf-1 functions downstream of c-HaRas.

H 501 CHARACTHERIZATION OF THE HUMAN GCF PROTEIN, A NEGATIVE TRANSCRIPTIONAL REGULATOR OF THE EGF RECEPTOR GENE.

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Laboratory of Molecular Biology, NIH, Bethesda Md. 20892.

The human EGF Receptor (EGF-R) is a proto-oncogene whose over-expression is implicated in the development of several types of human tumors. Transcriptional regulation of EGF-R gene is complex. The EGF-R promoter is GC rich and binds several transcription activators, like Sp1 and ETF. In addition, one repressor protein named GCF has been cloned and shown to bind to EGF-R promoter GC rich sequences and to repress its transcription *in vitro*. GCF is composed of a highly basic amino terminal region which constitute the DNA binding region, two leucine-zipper motifs and contains several putative nuclear localization signals. As an initial step to characterize GCF protein and to address its role we have developed antibodies against a bacterial expressed GCF-fusion protein. GCF antibodies recognize GCF protein synthetized *in vitro* and in cell extracts from human cells. In cell extracts, the major form of GCF has a molecular weight of approximately 97Kdalton. In addition other less abundant species with a slightly higher apparent molecular weight are specifically recognized, suggesting extensive post-translational modification. GCF is expressed both in EGF-R negative human cell lines (HUT-102, U266) and in EGF-R expressing cells (KB, A431, AGS). Cell fractionation studies indicate that the major form of GCF is predominantly localized in the nucleus. In addition, GCF is a phosphoprotein and the phosphorylated form is associated with the nuclear compartment both in HUT-102 and KB cells. These antibodies will provide an useful tool to further address the mechanism by which GCF regulates EGF receptor expression in normal and transformed human cells.

H 502 PLEIOTROPIC GROWTH DEFECTS ASSOCIATED WITH DELETION OF THE PHO85 GENE OF *SACCHAROMYCES CEREVISIAE*,

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The PHO85 gene product is a putative protein kinase required for the transcriptional repression of acid phosphatase (PHO5) production in yeast. We show that deletion of 31 amino acids internal to the PHO85 protein results in a null mutation coincident with constitutive expression of acid phosphatase. Strains containing this PHO85 deletion within the genome exhibit multiple defects, associated with growth of the cell. Strains lacking the PHO85 gene show reduced growth rate in medium containing glucose as a carbon source and fail to grow in medium containing glycerol/lactate. Interestingly, certain strains containing the deletion fail to grow in medium containing galactose as a carbon source. This suggests that the PHO85 gene plays a role in carbon metabolism and cell growth, in addition to its role in expression of PHO5. Mating studies have shown that the galactose defect is due to a single, unlinked (to PHO85) gene present in these strains. Using a plasmid library, we have cloned this gene, termed PGS1 (Phosphate-Galactose Suppressor). A molecular characterization of this gene is ongoing. The galactose growth defect appears to be due to the inability to express galactose-utilization genes and subsequently ferment galactose. This Gal- phenotype is not a general glucose-repression defect since expression of CYC1 and SUC2 are unaffected by the deletion of PHO85. Finally, diploids homozygous for the PHO85 deletion fail to sporulate.

H 503 ROLES OF JUN AND FOS FAMILY MEMBERS IN MODULATION OF AP-1 ACTIVITY AND TUMOR PROMOTION IN JB6 CELLS

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Mouse epidermal JB6 cells are genetic variants sensitive (P+) or resistant (P-) to promotion of transformation by a variety of tumor promoting agents including tetradecanoyl phorbol-13-acetate (TPA), epidermal growth factor (EGF), and other promoters. Previously we reported that P+ cells show TPA and EGF-inducible, AP-1 dependent transactivation of gene expression, whereas P- cells do not (Science 244, p.566). In the present investigation levels of several Jun and Fos family mRNAs and proteins were measured in P+ and P- cells. P+ and P- cells showed similar basal and TPA and EGF induced levels of Jun B mRNA and protein, and showed little evidence of differential Jun D induction. Basal levels of Jun D protein, and, more notably, mRNA were higher in P+ cells. In contrast tumor promoter induced as well as basal levels of c-Jun mRNA and protein were significantly higher in P+ Cl 41 cells than in P- Cl 30 cells. This was generalizable to the additional independent P+ and P- lines Cl 22 (P+) and Cl 25 (P-), but not to JB6 cells which had spontaneously acquired a P+ phenotype, suggesting that elevated c-Jun may be instrumental for, but not always required in the promotion pathway(s). Additionally, overexpression of c-jun in P- cells did not result in acquisition of P+ responsiveness, suggesting that elevated c-Jun expression is not sufficient for promotion sensitivity.

Upon measurement of c-Fos and Fra-1 levels, TPA and EGF-induced levels of both mRNAs were similar in P+ and P- cells. Measurement of Fos B protein levels revealed similar basal and induced levels of that protein, as well. However, in P- cells TPA and EGF induced distinctly higher levels of a form of Fra-1 protein that co-migrates with phosphorylated Fra-1 than was observed in P- cells. Furthermore, c-Jun, Jun B and Jun D protein levels underwent down-modulation as a function of increasing cell density, whereas Fra-1 protein underwent significant upregulation. These data suggest that c-Jun levels may in part play a role in differential transactivation and P+ responsiveness, that Fra-1 may function to suppress transformation by tumor promoting agents in P- cells, and that Fra-1 may additionally modulate negative growth control in JB6 cells.

H 504 CELL TRANSFORMATION BY JUN: THE ROLE OF HETERODIMERIZATION. Timothy J. Bos and Mark Hughes. Department of Microbiology and Immunology, Eastern Virginia Medical School, P.O. Box 1980, Norfolk, VA 23501.

Overexpression of c-Jun or v-Jun, in chicken embryo fibroblasts (CEF), will lead to cell transformation with varying efficiencies. The oncogenic mechanisms, however, still are not clear. C-Jun belongs to a family of proteins which require dimerization for activity. Both c-Jun and v-Jun are capable of forming heterodimeric complexes with other Jun family and Fos family members. In addition, c-Jun and v-Jun can form homodimers which appear to be less stable than heterodimers. Kovary and Bravo have shown that the relative proportion of each Jun family - Fos family complex is governed by the relative abundance of each at any one time. Presumably, each of the various Jun family - Fos family complexes exert subtle differences in DNA binding or transcriptional regulatory specificities. When c-Jun or v-Jun are overexpressed, they become the predominant species which increases the relative proportion of homodimers. Gel shift assays, with nuclear proteins isolated from v-Jun, c-Jun or vector infected CEF reveal a striking difference in the abundance of three protein complexes which recognize the consensus AP-1 site. A protein complex thought to consist of Jun homodimers predominates in v-Jun expressing CEF; is weaker in c-Jun expressing CEF and is absent in vector infected CEF. Interestingly, a complex which contains both Jun and Fos is greatly reduced in v-Jun expressing CEF compared to vector infected CEF. It thus appears that cell transformation may correlate with the relative abundance of Jun homodimers and Jun-Fos heterodimers. It is not clear what role, if any, the other Jun and Fos family proteins play during cell transformation. For this reason, we have examined the ability of Jun to induce cell transformation in CEF in the absence of interaction with other Jun or Fos family proteins.

To this end, we have constructed a chicken v-Jun mutant that is capable only of homodimerization. This was accomplished by replacing the leucine zipper region of Jun with that of the yeast transcription factor, GCN4. Expression of this protein in CEF will result in synthesis of a chimeric protein that will not heterodimerize with any of the endogenous Jun or Fos proteins but retains all of the DNA binding and transcriptional regulatory domains of Jun. This chimeric protein has been cloned into a retroviral expression vector (RCAS) and assayed for cell transformation by focus formation. In addition, the DNA binding specificities and dimerization specificities have been assayed using *in vitro* translated proteins. The results of these assays will be discussed.

H 505 EFFECT OF ANTICANCER DRUGS ON REGULATION OF GENE EXPRESSION: MODIFICATION OF THE BINDING OF TRANSCRIPTIONAL FACTORS TO DNA, M. Broggini, M. Cattabeni, S. Fabbri, C. Prontera, M. Bonfanti. M. D'Incalci. Mario Negri Institute, Milan, Italy.

Many anticancer agents bind to DNA with some degree of sequence specificity. The precise mechanism of action of these drugs is not known. We have analyzed if the binding of different alkylating and non-alkylating agents can interfere with the binding of transcriptional factors recognizing the same sequence of DNA. By using a gel shift assay we have shown that Distamycin A which recognizes AT rich sequences inhibits the binding of OTF-1 (that binds to an octamer ATGCAAAT) but not of a factor (GATA) which binds to a GC rich sequence. To study the effects of drugs alkylating 06-guanine, we have constructed modified oligonucleotides containing 06-methylguanine (06MeG) in the recognition sequence of the transcription factors NFkB, sp1 and the serum response element of the c-fos promoter. The presence of 06MeG in crucial sites of the sequence is able to inhibit the binding of these factors. Similar results were obtained when unmodified oligonucleotides are pretreated with N7-guanine alkylators.Two compounds, quinacrine and nitrogen mustards, which have a different specificity in the binding to DNA, have shown a different ability to block the binding of transcription factors to DNA in vitro. In addition, the ability of these two compounds to inhibit gene expression was evaluated by using a reporter gene (CAT) under the control of promoters containing different binding sites for transcription factors. A good correlation between inhibition of transcription factors binding to DNA (measured in vitro) and the level of gene expression (measured in the cells) was found. These results suggest that some alkylating anticancer agents might act through the block of the binding of transcriptional factors to DNA and possibly modifying the regulation of the expression of specific genes.

H 506 SEQUENCES INVOLVED IN THE REGULATION OF THE RAT PLASMINOGEN ACTIVATOR INHIBITOR (PAI-1) GENE: EVIDENCE FOR BOTH SIMPLE AND COMPLEX GLUCOCORTICOID RESPONSE ELEMENTS, Carolyn J. Bruzdzinski, Maureen R. Johnson, Sigal S. Winograd and Thomas D. Gelehrter, Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109-0618

Glucocorticoids regulate tissue-type plasminogen activator (tPA) activity in HTC rat hepatoma cells primarily by modulating plasminogen activator-inhibitor (PAI-1) gene expression. To investigate the molecular mechanisms underlying this regulation, we have cloned the rat PAI-1 gene from an HTC genomic library. Multiple putative glucocorticoid response elements (GREs) were identified within 2.4 kb of 5' flanking sequence. Analyses of a series of hybrid genes, containing various portions of the 5' flanking region of the rat PAI-1 gene fused to the chloramphenicol acetyl transferase reporter gene, transfected into HTC rat hepatoma cells localized the region involved in the transcriptional regulation by glucocorticoids to between -1230 and -760. This region contains a sequence that is >90% identical to the consensus GRE 15-mer. The sequence binds purified glucocorticoid receptor DNA binding domain protein (GR-DBD), as demonstrated by both gel mobility shift assays and DNAse I footprinting. Point mutations of this GRE within the context of the rat PAI-1 promoter reduced the glucocorticoid induction by 60-75% when analyzed by transfection of HTC cells and abolished the binding of GR-DBD in gel mobility shift assays, confirming that this GRE mediates the glucocorticoid response of the rat PAI-1 gene in HTC cells.

In HepG2 cells, a human hepatoma cell line, neither the endogenous PAI-1 gene nor the transfected rat PAI-1 promoter is induced by glucocorticoids. However, when it is co-transfected with a glucocorticoid receptor expression vector, the rat PAI-1 promoter is induced 10-fold. Two regions are responsible for this induction: the GRE at -1196 and a second region between 760 and 270 bp upstream of the start site of transcription. This area contains >200 bp which are >80% identical with a region in the human gene that is involved in the glucocorticoid regulation of that gene, but does not contain a putative GRE 15-mer. Since this region is not active in response to glucocorticoids when the rat PAI-1 promoter is transfected into HTC cells, the underlying mechanism may be more complex, involving not only the glucocorticoid receptor, but other auxiliary transacting protein(s), not present in HTC cells.

H 507 Cooperativity between multiple elements which mediate phorbol ester induction and glucocorticoid repression of human urokinase promoter.

Anna Caracciolo, Claus Nerlov, Fabrizia Pergola, Dario de Cesare, Francesco Blasi, Morten Johnsen and Pasquale Verde, University Institute of Microbiology, Øster Farimagsgade 2A, 1353 Copenhagen K, Denmark and International Institute of Genetics and Biophysics, Via Marconi 12, 80125 Naples, Italy.

Urokinase is a serine protease controlling a proteolytic cascade important for regulation of peri- and extracellular proteolysis both in the normal organism and during tumor invasion. The urokinase gene is regulated by enhancer sequences located about 2kb upstream of the transcription initiation start site. This enhancer contains AP-1 and PEA3 binding sites necessary for both basal level and induced transcription mediated by the enhancer (1,2). We have found that a region of the enhancer situated between two AP-1 sites is required for the cooperation of AP-1 and PEA3 during TPA-induction of uPA transcription in HepG2 human hepatoma cells. This region contains several evolutionally conserved binding sites for nuclear proteins. Mutation of individual binding sites reduces the TPA-inducibility of the enhancer, and mutation of all of them abolishes it completely.

Investigation of the regulation of uPA expression by glucocorticoids showed that the same enhancer sequences were required for regulation by these effectors.

These observations clearly indicate that the PEA3 and AP-1 binding elements in the uPA enhancer are of crucial importance to the transcriptional regulation of the uPA gene, and that additional sequence elements are involved in mediating their activity.

H 508 IDENTIFICATION OF USF AS THE UBIQUITOUS MURINE FACTOR THAT BINDS TO AND STIMULATES TRANSCRIPTION FROM THE IMMUNOGLOBULIN λ2-CHAIN PROMOTER, Ling A. Chang[1], Tammy Smith[1], Philippe Pognonec[2], Robert G. Roeder[2] and Helios Murialdo[1], [1]Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8 Canada, [2]Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10021

To study the specificity and identity of NF-λ2, a ubiquitous murine nuclear factor that interacts specifically with the promoter of the λ2-chain gene and stimulates its transcription, competition experiments were carried out using DNA fragments from various immunoglobulin regulatory elements. The results showed that a fragment containing the H-chain enhancer competed efficiently for the binding of NF-λ2. Dissection of the H-chain enhancer revealed that the *μE3* motif contributed the competing ability. Additionally, a regulatory region found in the adenovirus major late promoter, which interacts with the human general transcription factor USF, competed very efficiently for binding to NF-λ2. This region contains a sequence, **CACGTGAC,** which is identical to a region within the *NF-λ2* motif. The pattern of complexes formation using oligonucleotide probes corresponding to the *NF-λ2* and *USF* motifs were identical, and they both differed from that displayed by the E3 probes.

Antisera against different domains of USF also react specifically with NF-λ2 showing that this factor is antigenically related, if not identical, to USF. Furthermore, the activity of the λ2 promoter in an *in vitro* transcription assay was significantly reduced when the nuclear extract used was USF-depleted. Addition of exogenous USF to this extract restored the transcription activity. Therefore, we conclude that NF-λ2 is the murine homologue of USF.

H 509 IDENTIFICATION OF MAMMALIAN LEUCINE ZIPPER PROTEINS THAT REACT WITH JUN AND FOS BY PROTEIN INTERACTION CLONING IN YEAST, Pierre Chevray and Daniel Nathans, Department of Molecular Biology and Genetics and Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.
Jun and Fos are sequence-specific DNA-binding transcriptional regulators of the bZip class. Members of these families form homo- or heterodimers mediated by the leucine zipper structural motif and bind to DNA via basic regions adjacent to the dimerizing domains. All pairwise combinations of Jun and Fos family members capable of forming stable dimers bind to the same dyad symmetric core sequence known as an AP-1 site.

To identify proteins that form heterodimers with Fos or Jun that might have novel DNA-binding specificities we have extended the yeast protein interaction cloning system developed by Fields and Song (Nature 340:245-246, 1989) to clone mammalian DNAs that encode polypeptides that interact with the leucine zippers of Jun and Fos. For this purpose yeast cells expressing a GAL4 (DNA binding domain) - c-Jun (bZip) fusion gene were transfected with a mouse embryo cDNA library fused to GAL4 (activation domain) codons, and transformants showing GAL4 activity were selected. In this way several cDNAs have been identified that code for polypeptides expected to form coiled-coil structures with Jun and/or Fos. One of these is a bZip protein of the ATF family; its interaction with Fos and Jun and DNA binding properties are now being studied. Our results illustrate the range of protein interaction cloning for discovering proteins that bind to any given target polypeptide.

H 510 REGULATION OF GENE EXPRESSION BY YEAST MATING PHEROMONES, Brent H. Cochran, Niranjan Pandey, Erica Marsilio, and Piali Sengupta. Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Room E17 - 517, 77 Mass. Ave., Cambridge, MA 02139.

We have previously identified a factor in *S. cerevisiae* that binds specifically to the human c-*fos* serum response element (SRE). This factor is encoded by the *MCM1* gene in yeast and shares considerable sequence homology to the mammalian serum response factor (SRF). We have shown that the MCM1 protein, like the mammalian SRF, functions in a signal transduction pathway in yeast that leads from the cell surface to the nucleus. A 26 bp sequence upstream of the *STE3* gene is sufficient to confer **a**-factor inducibility onto a heterologous gene. This sequence contains the binding sites (P+Q boxes) for MATα1 and MCM1. We have examined the roles of α1 and MCM1 in the response to **a**-factor in α cells. Gene fusions have been used to investigate whether induction conferred by the PQ box is mediated through either MATα1 or MCM1, or a combination of both. When MCM1 is fused to the DNA binding domain of the bacterial repressor LexA, this fusion protein is capable of transactivating a *lacZ* reporter gene driven by a LexA operator. However, the transcriptional activity of the MCM1-LexA fusion is not further enhanced by treatment of cells with **a**-factor. A MATα1-LexA fusion protein is also capable of transactivation through a LexA operator. Moreover, the activity of the MATα1-LexA fusion protein can be further induced upon treatment with **a**-factor. However, the activity of the MATα1-LexA fusion protein is dependent on the functions of genes in the pheromone signal transduction pathway including *STE7*, *STE11*, and *STE12* genes. Since MCM1 shares sequence and functional homology with mammalian serum response factor (SRF) and MATα1 is analogous to the mammalian protein p62^{TCF}, it is likely that these results also have implications for the mechanisms of regulation of the c-*fos* proto-oncogene in mammals. Moreover, we show that human SRF can partially complement mutations in the *mcm1* gene.

H 511 THE RAS ONCOGENE INCREASES THE POTENCY OF THE TRANS-ACTIVATION DOMAIN OF C-MYC. Michael S. Colman and Michael C. Ostrowski, Department of Microbiology & Immunology, Duke University Medical Center, Box 3020, Durham, NC, 27710.
The classic example of oncogene collaboration in cellular transformation is the requirement for both activated ras and c-myc for transformation of primary rodent embryo fibroblasts. This collaboration might indicate that ras and myc are in separate signal transduction pathways both required for transformation, or it may indicate that both are in the same pathway and ras is required to fully trigger myc activity. We have recent evidence suggesting that the latter may be the case. In particular, we can demonstrate that ras can increase the effectiveness of the weak trans-activation domain located in the N-terminal portion of c-myc by 15-20-fold in transient transfection assays. Evidence concerning the biochemical basis of this activation and its biological consequences will be presented.

H 512 TRANSCRIPTIONAL ACTIVATION BY THE MOUSE ESTROGEN RECEPTOR, P.S. Danielian, R. White, S.A. Hoare, J. A.Lees and M. G. Parker, Imperial Cancer Research Fund, London, England. The estrogen receptor is a member of the nuclear hormone receptor superfamily of transcription factors. The ability of the receptor to regulate gene expression is dependant on the high affinity binding of a hormonal ligand. In the presence of estradiol the receptor appears to undergo a process of 'transformation'. This involves the dissociation of hsp90 allowing receptor dimerisation, high affinity DNA binding to hormone response elements and the generation of a ligand dependant transcriptional activation function, TAF-2.

It has been demonstrated that in the mouse estrogen receptor a region near the C-terminus of the protein required for ligand binding overlaps with residues forming an essential part of the dimer interface (1). We have now identified a second region within the hormone binding domain which is essential for TAF-2 activity and shown that these sequences are conserved in the nuclear hormone receptor superfamily. Specific point mutations have been introduced into the receptor and the proteins generated fail to stimulate transcription in a transient transfection assay yet retain steroid binding and high affinity DNA-binding. The N-terminal region of the receptor also contains a transciptional activation function TAF-1, the activity of which in transient transfection varies depending on either the cell type transfected or the steroid responsive promoter used. The analysis of mutant receptors demonstrates that for some mutants an effect on transcriptional activation is observed only in the absence of TAF-1, suggesting that TAF-2 can cooperate with sequences in the N-terminal region of the receptor. Mutation of the conserved residues in the mouse glucocorticoid receptor results in a loss of stimulation of transcription indicating that this region may be essential for the hormone dependent transcription by other members of the nuclear receptor family.

(1) S. E. Fawell *et al* Cell 60 953-962 (1990).

H 513 HUMAN TGF-α CONTAINS AN ESTROGEN RESPONSIVE ELEMENT COMPOSED OF TWO IMPERFECT PALINDROMES. D. El-Ashry, M.E. Lippman, and F.G. Kern. Lombardi Cancer Center, Georgetown University, Washington, DC 20007. Estrogen treatment of human breast cancer cells results in a 2-3 fold induction of transforming growth factor-α (TGF-α) message and a 2-5 fold induction of the protein. The TGF-α promoter contains no consensus estrogen response element (ERE), but does contain two imperfect palindromes separated by 20 bp. To determine whether this putative ERE is capable of responding to estrogen, we examined its ability to mediate estrogen induction of chloramphenicol acetyl transferase (CAT). The putative ERE was synthesized as a 58-mer and inserted into a CAT expression vector in place of the glucocorticoid response element of a mouse mammary tumor virus promoter. This vector was assayed for estrogen-inducible CAT expression by transient co-transfection with an estrogen receptor (ER) construct into COS-7 cells and MCF-7 human breast cancer cells. In the absence of estrogen, only low basal CAT activity was observed, while estrogen treatment resulted in a 35-fold induction in both cell types. Gel shift assays using ^{32}P-end labeled 58-mer and human ER resulted in upshifted bands, two of which could be further shifted with an anti-ER monoclonal antibody indicating the presence of ER. The activity of the 58 bp element was then examined in the context of its own promoter. Co-transfection of a CAT construct consisting of 1100 bp or 330 bp of TGF-α promoter sequence and an ER construct resulted in only a 2-5 fold induction of CAT by estrogen. **Conclusions**: 1) the human TGF-α promoter contains an ERE-like sequence that mediates substantial estrogen induction (35-fold) through a heterologous promoter and interacts specifically with ER; 2) this sequence in the context of its own promoter mediates an estrogen induction of 2-5 fold, consistent with the levels of TGF-α message and protein induced by estrogen in human breast cancer cells; and 3) these differences may reflect the presence of a repressor binding site within the TGF-α promoter or a reduction in the affinity of ER binding due to the interaction of other transcription factors with nearby sites.

H 514 **Nuclear Association of a Transcription Factor Essential for T cell Activation is blocked by Cyclosporin A and FK506.** W. Michael Flanagan, Blaise Corthésy, Richard J. Bram and Gerald R. Crabtree. Howard Hughes Medical Institute. Stanford University Medical School. Stanford, CA 94305.

In T lymphocytes, cyclosporin A (CsA) and FK506 disrupt the transmission of signals from the T cell antigen receptor to cytokine genes that coordinate the immune response. Although the molecular mechanism by which CsA and FK506 exert their immunosuppressive properties is unknown, these drugs do not interfere which the known membrane events involved in triggering the antigen receptor. The putative intracellular receptors for FK506 (FK binding protein) and CsA (cyclophillin) are cis-trans prolyl isomerases. Binding of the drug inhibits isomerase activity; however, studies with other prolyl isomerase inhibitors and analysis of CsA/FK506-resistant mutants in yeast suggest that the effects of the drug result from the formation of an inhibitory complex between the drug and isomerase and not inhibition of the isomerase activity. Recent data indicates that calcineurin, a calcium-activated, serine-threonine phosphatase, specifically binds to the drug isomerase complex resulting in the alteration of its enzymatic activity.

A transcription factor, NF-AT, essential for early T cell gene activation, appears to be a specific target of CsA and FK506 since transcription directed by this protein is completely blocked in T cells treated with these drugs, with little or no effect on other transcription factors such as AP-1 and NF-kB. We find that NF-AT is formed when a Ca^{++} signal emanating from the antigen receptor induces a pre-existing cytoplasmic subunit to translocate to the nucleus and combine with a newly synthesized nuclear subunit of NF-AT. FK506 and CsA block translocation of the cytoplasmic component without affecting synthesis of the nuclear subunit.

H 515 PHORBOL ESTER-INDUCED AMINO TERMINAL PHOSPHORYLATION OF C-JUN BUT NOT JUNB REGULATES TRANSCRIPTIONAL ACTIVATION, Christopher C. Franklin*, Veronica Sanchez*, Fred Wagner*, James R. Woodgett#, and Andrew S. Kraft*, The Division of Hematology/Oncology, University of Alabama at Birmingham, Birmingham, Alabama 35294* and Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT#

Phorbol ester tumor promoters activate gene transcription by regulating both the synthesis and post-translational modification of the AP-1 family of transcription factors. c-Jun and JunB are both components of the AP-1 complex and are capable of inducing AP-1 responsive genes by binding to the tumor-promoter response element (TRE). However, c-Jun is a more potent transcriptional activator than JunB, and cotransfection assays demonstrate that JunB inhibits the activity of c-Jun. Here, we demonstrate that phorbol esters stimulate the phosphorylation of the amino terminal transcriptional activating domain of c-Jun, but not JunB. Mutational analysis indicates that this phosphorylation is occuring on serines 62 and 72 *in vivo*. Using a fusion of the GAL4 DNA binding domain to the N-terminal 89 amino acids of c-Jun, we demonstrate that this region is sufficient for transcriptional activation. Transcriptional activation of this fusion protein by phorbol esters is completely abrogated by mutation of serines 62 and 72 to leucines. In contrast to c-Jun, when fused to GAL4 the identical amino terminal region of JunB does not stimulate transcription when cells are treated with phorbol esters. These results demonstrate that the differential regulation of inducible transcription by members of the Jun family, c-Jun and JunB, may occur as a consequence of amino terminal phosphorylation.

H 516 Phosphorylation of the Human c-myc Protein by Nuclear Kinases, J.A. Frost and J.R. Feramisco, Departments of Pharmacology and Medicine, UCSD Cancer Center, 9500 Gilman Drive, La Jolla, CA 92093-0636

In recent years the phosphorylation of transcription factors has been shown to be important in regulating their activities. For example, transactivation by the CREB protein is contingent upon its phosphorylation. Similarly, the ability of factors such as c-jun, SRF, and c-myb to bind their cognate DNA sequences in vitro is profoundly altered by phosphorylation. Recent evidence suggests that the c-myc proto-oncogene is involved in regulating gene expression. In addition, previous work has shown the protein to be phosphorylated at a number of sites in vivo, a subset of which can be phosphorylated in vitro by purified casein kinase II. In an effort to identify other kinases that may phosphorylate c-myc, we have analysed the phosphorylation of a purified recombinant human c-myc protein in vitro by fractionated rat kidney fibroblast nuclear extracts. Four peaks of c-myc kinase activity were identified, two of which were clearly serum inducible. All four activities phosphorylate c-myc primarily on serine residues. The two constitutive activities are inhibited by low concentration of heparin and can use GTP as a phosphoryl donor, suggesting that they may be differentially phosphorylated forms of casein kinase II. All four activities phosphorylate c-myc primarily on serine residues. The two constitutive activities are inhibited by low concentrations of heparin and can use GTP as a phosphoryl donor, suggesting that they may be differentially phosphorylated forms of casein kinase II. All for activities exhibit distinct affinities for substrates such as myoD, c-jun, p53, casein histones I, II, and V, and myelin basic protein. Current efforts are aimed at identifying the kinases involved and determining which phosphorylate the c-myc protein on sites found in vivo.

H 517 A NOVEL JUND-ASSOCIATED DNA BINDING COMPLEX IN T-CELLS, Kevin Gardner, Terri Davis-Smyth, Antonietta Fariana, Henry Krutzsch, T. Carlton Moore, and David Levens, Laboratory of Pathology, National Cancer Institute, National Institutes of Health, Bethesda, MD. 20892

The major AP1 binding complex in the gibbon T-cell line, MLA 144, is composed primarily of JunD. This cellular JunD associated complex has been purified to homogeneity and has been found to be highly dependent on a separate activator protein(s) for DNA binding. This activator protein(s) has been partially purified and found to be composed of 25 and 27 kd polypeptides that, by gel retardation analysis, dramatically increase (as much as 100 fold) the binding of cellular JunD to oligonucleotides containing the canonical AP1 consensus sequence. Interestingly, in vitro translated recombinant JunD is minimally responsive to the activator by gel retardation analysis and migrates at a slower mobility. Likewise, neither recombinant c-jun nor Hela derived c-fos/c-jun complexes show comparable enhancement (no greater than 6 fold) of binding by partially purified or gel purified activator.

Copper cleavage footprint analysis of the interaction of the cellular JunD complex with DNA reveals extensive contacts outside of the AP1 consensus sequence that are distinct from those made by c-Jun. Footprint analysis of the interaction of partially purified activator with DNA demonstrates extensive contacts with DNA that overlap those made by the purified cellular complex.

Preliminary in vitro transcription analysis indicates that both the purified T-Cell AP1 binding complex and the isolated activator enhance transcription singly or in combination. Additional studies elucidating the mechanism of DNA binding enhancement and the possible role of post-translational modification in the modulation of JunD activity are discussed.

H 518 TRANSCRIPTIONAL CONTROL OF c-fos EXPRESSION BY CASEIN KINASE II AND CA^{2+}/PHOSPHOLIPID-DEPENDENT PROTEIN KINASE, Cécile Gauthier-Rouvière, Anne Fernandez and Ned J.C. Lamb, Cell Biology, CRBM-CNRS-INSERM, BP5051, 34033 Montpellier Cedex France.

One of the genes induced during mitogenic activation of quiescent cells is the protooncogene c-fos. The c-fos promoter contains different sequences involved in its expression through the interaction of different DNA binding phosphoproteins.

We have examined the potential of two purified protein kinases, the casein kinase II (CKII) and the Ca^{2+}/phospholipid-dependent protein kinase (C-kinase) to induce c-fos expression. Both kinases induce the rapid expression of c-fos when injected into quiescent REF-52 cells. Coinjection of SRE oligonucleotide inhibits the induction of c-fos by both kinases whilst TRE oligonucleotide inhibits only c-fos induced by C-kinase. Moreover, c-fos expression induced by either CKII injection or serum activation is inhibited by microinjection of antibodies against $p67^{SRF}$, a nuclear phosphoprotein that binds to SRE. This protein is rapidly phosphorylated after serum addition of CKII injection suggesting that CKII (as serum) induces c-fos expression through binding of phosphorylated $p67^{SRF}$ at the SRE sequence.

We also observed that $p67^{SRF}$, in addition to its role at the G_0 to G_1 transition is involved in the G_1-S transition. Indeed, impairing of $p67^{SRF}$ activity through microinjection of anti-$p67^{SRF}$ antibodies or SRE oligonucleotides after the G_0-G_1 transition inhibits DNA synthesis implying that $p67^{SRF}$ is an integral component of mammalian cell transcriptional control.

H 519 SEQUENCE-SPECIFIC TRANSCRIPTIONAL ACTIVATION BY MYC AND REPRESSION BY MAX. Nissim Hay[1,2], Chirag Amin[1,3] and Andrew Wagner[1,4]: The Ben May Institute[1] and the Departments of Pharmacological and Physiological Sciences[2], Molecular Genetics and Cellular Biology[3] and Biochemistry and Molecular Biology[4], University of Chicago, Chicago IL 60637.

The identification of the specific cognate DNA binding site for MYC protein (1) and the isolation of its specific heterodimeric partner, MAX (2,3), enabled us to study the function of MYC as a transcriptional regulator and how this function is modulated by MAX. The activity of MYC and MAX as sequence-specific transcriptional regulators was evaluated by employing an *in vivo* transient transfection and CAT assay. The reporter plasmids contained the minimal rat prolactin promoter with tandem repeats of the consensus MYC binding sites upstream of it, linked to the CAT gene.

The reporter plasmids were transfected into Ltk- cells along with increasing amounts of c-*myc* expression vector. Cotransfection of MYC expression vector leads to approximately 5 fold increase in CAT activity from plasmids that contain the AdML-USF and the EMS (3) binding sites but not from the plasmid that contains the TFE3 binding sites. Analysis of MYC mutants in the same assay revealed that the integrity of both the HLH and LZ domains are required for transactivation; as is the basic domain adjacent to the HLH domain, whereas the non-specific DNA binding domain is not required for transactivation. The N-terminus region of MYC that is required for transformation is also required for transactivation; replacing this region with the VP16 activator domain elicits further transcriptional activation. From these results we concluded that MYC is able to bind its cognate DNA binding site *in vivo* and to transactivate transcription through this site. In contrast to MYC, overexpression of MAX along with the reporter plasmids results at least 10 fold decrease in CAT activity from plasmids that contain the consensus MYC binding sites. Replacing the C-terminus of MAX with the VP16 activator domain converts MAX to a transcriptional activator. Coexpression of MAX and MYC inhibits the transactivation by MYC. Our results suggest that MAX may modulate MYC activity in an antagonistic fashion. Indeed, we found that MAX is expressed at higher levels than MYC in growth arrested and differentiated cells, whereas MYC is expressed at higher levels in proliferating cells. A model for the activity of MYC and MAX in the cells will be discussed.

1. Blackwell, T. K. et al. (1990) Science 250, 1149-1151
2. Blackwood, E. M. and Eisenman, R. N. (1991) Science 251, 1211-1217
3. Prendergast, G. C. et al. (1991) Cell 65, 395-408

H 520 LRF-1 and junB MEDIATED TRANSCRIPTIONAL ACTIVATION AND REPRESSION; IMPLICATIONS FOR THE EARLY GROWTH RESPONSE IN REGENERATING LIVER Jui-Chou Hsu and Rebecca Taub Department of Human Genetics, HHMI U. Penn. School of Medicine, Phila., PA 19104

Understanding transcriptional responses during liver regeneration is of major importance in understanding the regulation of cell growth. We have previously described the identification of a novel, abundant immediate-early gene encoding a 21kD leucine-zipper containing protein, that we have designated LRF-1 (liver regeneration factor), a member of the jun-fos family of transcription factors. In regenerating liver and a mitogen-stimulated liver cell line, LRF-1, c-fos, junB and c-jun mRNAs are highly induced, but fra-1 and fosB mRNAs are not induced. In mitogen-stimulated fibroblasts, all six jun-fos family members are induced, but LRF-1 is induced to a lesser extent. We investigated the role of LRF-1 and other fos-jun family members present during the hepatic and fibroblast growth response. Using specific antibodies, we detected specific LRF-1/jun protein complexes during the early growth response of hepatic cells. To demonstrate the role of LRF-1 in the early growth response we performed transient transfections using LRF-1, c-jun, junB and c-fos expression vectors in serum-deprived NIH-3T3 cells. We have shown that LRF-1 in combination with either junB or c-jun strongly activates a CRE-cat reporter gene which fos/jun does not activate. On the other hand, LRF-1 in combination with c-jun activates, while LRF-1 in combination with junB strongly represses AP-1-cat reporters. This is in contrast with c-fos which strongly activates AP-1-cat reporters in combination with both c-jun and junB. Using deletion mutant analysis, we have identified a repressor domain in LRF-1 that is responsible for LRF-1/junB mediated repression of AP-1 elements. Temporally, LRF-1 is expressed later than c-fos during liver regeneration, and may function to repress transcription of fos-induced genes containing AP-1 sites in their promoters. LRF-1 may activate transcription of the many liver-specific genes whose promoters contain CRE elements. In this way LRF-1 may have a unique and important role in growth regulation in regenerating liver.

H 521 CHROMOSOMAL TRANSLOCATION t(15;17) IN HUMAN ACUTE PROMYELOCYTIC LEUKEMIA FUSES RARα WITH A NOVEL PUTATIVE TRANSCRIPTION FACTOR, PML

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Karyotypic changes, including chromosomal translocation, are consistently found in certain types of cancer. The balanced t(15;17) (q21-q11-22) translocation is exclusively associated with acute promyelocytic leukemia (APL), and it is often the only visible karyotypic aberration present. This translocation is detected in as many as 90% of APL patients and has become the definitive marker for the disease. The chromosome 17 breakpoint in APL was recently mapped to the *retinoic acid receptor(RAR)* α gene, and the translocation results in a fusion between *RARα* and a region on chromosome 15 referred to as PML(ProMyeLocytes). PML contains a cysteine-rich region present in a new family of apparent DNA-binding proteins that includes a regulator of the interleukin 2 receptor gene (Rpt-1) and the recombination activating gene product (RAG-1). Accordingly, PML may represent a novel transcription factor or recombinase. The aberrant PML-RAR fusion product, while typically retinoic acid responsive, displays both cell type and promoter specific differences from the wild type RARα. Because patients with APL can be induced into remission with high dose RA therapy, we propose that the nonliganded PML-RAR is a new class of dominant negative oncogene product. Treatment with RA would not only relieve this inhibition but the activated PML-RAR may actually promote myelocyte differentiation.

H 522 REGULATION OF THE PCNA PROMOTER BY THE ADENOVIRUS E1A 12S PROTEIN, Chitra Kannabiran and Michael B. Mathews, Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724

The adenovirus E1A gene is capable of transforming cells. As part of its transforming activity, it causes cells to overcome normal growth regulation and thereby acquire the ability for uncontrolled growth. Conserved regions 1 and 2, common to both the 12S and the 13S E1A gene products, are required for transformation.

The proliferating cell nuclear antigen, PCNA, is a DNA replication factor whose expression is required for DNA synthesis. The expression of PCNA is induced by E1A and the 12S E1A gene product is sufficient for this induction as shown by adenovirus infection of baby rat kidney cells and by co-transfection of HeLa cells with the PCNA-promoter driven CAT gene and a 12S expression vector. We are attempting to map the domains in the E1A 12S protein that are involved in induction of the PCNA promoter in HeLa cells. Our approach employs transient expression directed by a truncated PCNA promoter consisting of sequences from -87 to +60, the minimal promoter required for transactivation by 12S. Wild-type and mutant 12S constructs are expressed under the control of the cytomegalovirus early promoter in order to get high levels of expression. Results indicate that deletion of sequences within the N-terminal 85 amino acids results in loss of transactivation. A point mutant in conserved region 2, pm928, which is defective for transformation and binding to the retinoblastoma gene product, however, transactivated like wild-type 12S. This implicates sequences in the N-terminal non-conserved region and conserved region 1 of E1A in induction of PCNA. Studies are in progress to examine PCNA regulation in rodent fibroblast cells, which exhibit a greater degree of growth-regulation.

H 523 NUCLEAR TARGET OF CYCLOSPORIN A AND FK506 ACTION IS SPECIFICALLY BOUND BY A HETERODIMERIC PROTEIN COMPRISING MOLECULAR WEIGHTS 90K AND 45K. Peter N. Kao, Blaise Corthesy and Gerald Crabtree, Howard Hughes Medical Institute and Department of Medicine, Stanford University, Stanford CA 94305.

The immunosuppressants cyclosporin A and FK506 block the generation of a T-cell specific nuclear transcription factor: Nuclear Factor of Activated T-cells (NF-AT). The binding site for NF-AT is derived from the IL-2 enhancer (-285 to -255 bp); we have used the electrophoretic mobility shift assay to purify a nuclear protein that specifically binds the NF-AT sequence. Nuclear extracts from activated Jurkat T-cells were fractionated by sequential ion-exchange chromatography followed by a final purification over a sequence-specific DNA affinity column. The protein isolated retains binding to the labeled NF-AT site and is resistant to competition by a 50-fold molar excess of mutant oligonucleotide. The purified protein consists of 2 subunits of molecular weights 90K and 45K in an apparant 1:1 stoichiometry. These results are consistent with those of Flanagan et al. (Nature **352**:803 (1991)) who describe formation of NF-AT DNA-binding activity upon mixing non-activated cytoplasmic with activated nuclear extracts.

H 524 **Differential Expression of *Xenopus* Max, XMax, Genes**, Michael W. King, Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, THCMS, Terre Haute, IN. 47809

Expression of the *Xenopus* homolog of Max, XMax, occurs from at least two distinct genes. The predominant XMax protein is 24 amino acids smaller than the mammalian Max proteins making it only 136 amino acids and exhibiting a size of 19 kDa. A lesser abundant XMax protein contains an insertion, relative to XMax136, of 27 amino acids (XMax163) and exhibits a size of 21 kDa. Aside from the 27 amino acid insertion both XMax136 and XMax163 are identical. Both XMax proteins are highly conserved with the mammalian proteins, differing by only 12 amino acids. Only one of these differences resides in the bHLH-Zip domain. The insertion in XMax163 occurs at the C-terminal end of the Zip domain. Both species of XMax contain the 9 amino acid insertion domain near the amino terminus, yet, the sequences are poorly conserved with mammalian Max. Six of the 12 differences between XMax and mammalian Max are in this 9 amino acid domain. As yet no clones have been characterized that lack these 9 amino acids. The XMax proteins likely arise from distinct genes and not from an alternative splicing event since PCR amplification of cDNA and genomic DNA across the site of the 27 amino acid insertion yields identical products indicating there are no introns in this region of the XMax163 gene. The 27 amino acid insertion domain has several interesting features. There is the presence of a *cdc2* kinase recognition site not present in XMax136 nor other Max proteins. The sequences also are proline rich (19%). The level of XMax163 is much lower than XMax136 and is differentially expressed. Whereas, XMax136 expression essentially parallels that of c-Myc, XMax163 expression is distinct. The expression differences and sequence differences between XMax136 and XMax163 suggest they may exhibit different functions in *Xenopus*.

H 525 ISOLATION AND CHARACTERIZATION OF cDNA CLONES FOR N-*cym*, A GENE ENCODED BY THE DNA STRAND OPPOSITE TO N-*myc*. Geoffrey Krystal & Barbara Armstrong, Depts. of Medicine & Microbiology, Medical College of Virginia and The McGuire V.A. Medical Center, Richmond, VA 23249.

Bidirectional transcription from the N-*myc* oncogene locus in small cell lung cancer and neuroblastoma cell lines gives rise to naturally occurring sense and antisense transcripts. In addition to the nonpolyadenylated antisense RNA which forms a duplex with sense RNA retaining intron 1, antisense transcription also results in the formation of 0.9 and 1.8 kb polyadenylated RNAs. An analysis of cDNA clones derived from a neuroblastoma library reveals that the 0.9 kb RNA is composed of three exons, with the first two located within intron 1 one of the N-*myc* gene and the third located 5' of the major N-*myc* transcriptional initiation sites. This mRNA, which we have designated N-*cym*, is over-expressed in cell lines in which the N-*myc* locus is amplified, but is also detectable by PCR in fetal RNA samples. It can encode a 109 amino acid protein which shows no significant sequence homology to any protein in current data banks. However, secondary structure analysis reveals an amphipathic helix-loop-helix with an adjacent basic region, which shows no homology to characterized B-HLH proteins. N-*myc* and N-*cym* appear to be co-regulated in tumor cell lines under basal growth conditions and in response to the differentiating agent retinoic acid, suggesting that their protein products may be functionally interrelated during normal development and oncogenesis. We are currently directly addressing this question in co-transfection experiments, as well as determining the interactions between isolated protein products.

H 526 REGULATION OF TGFα GENE TRANSCRIPTION BY GLUCOSE AND GLUCOSAMINE IN VASCULAR SMOOTH MUSCLE CELLS, Jeffrey E. Kudlow, Donald A. McClain, Andrew J. Paterson and Mark Roos, Departments of Medicine and Cell Biology, University of Alabama at Birmingham, Birmingham, AL 35294

Accelerated atherosclerosis is a major complication of diabetes mellitus. Vascular smooth muscle cell proliferation is a precursor of atherosclerosis which could be triggered by abnormal growth factor expression in the diabetic milieu. For this reason, we investigated the effects of glucose and insulin on the transcription of the gene for one growth factor found in vascular smooth muscle cells, transforming growth factor-α (TGFα). We used firefly luciferase as a reporter gene linked to segments of the 5'-flanking region of the TGFα gene that we have recently characterized as the promoter for this gene. Glucose at 30 mM, but not insulin, stimulated transcription of the TGFα-luciferase construct 2-fold in transiently transfected early passage vascular smooth muscle cells. The effect was apparent by 30 min and was maximal at 12-18 hours. Glucosamine was much more potent than glucose in this regard, with a 12- to 15-fold stimulation seen with 5 mM glucosamine. These results in transfected cells were mirrored by equivalent effects of sugars on the levels of the endogenous TGFα mRNA in these cells. Pharmacologic experiments to inhibit protein kinase C activity did not block the effect of sugars on this gene, suggesting, that unlike other regulators of TGFα expression that rely on protein kinase C, the effect of the sugars is mediated by a distinct pathway. By deletional analysis of the TGFα gene, we have mapped the sugar responsive region of the gene to a 130 bp proximal segment. This segment of the gene contains 3 Sp1 consensus binding sites but the sugars do not regulate Sp1 mRNA, suggesting that the effect of the sugars occurs independent of the rate of Sp1 gene transcription. Our experiments suggest that glucose regulates TGFα gene transcription via the intermediary metabolite, glucosamine. This novel mechanism of growth factor gene regulation is likely to be representative of other growth regulatory genes and may be relevant in the pathogenesis of diabetic complications.

H 527 DIFFERENTIAL EXPRESSION OF IMMEDIATE EARLY TRANSCRIPTION FACTORS IN HUMAN LUNG CANCER, Wendy J. Levin[1] Richard B. Gaynor[2], Michael F. Press[3], Vikas Sukhatme[4] and Dennis J. Slamon[1]. [1]Division of Hematology-Oncology, UCLA School of Medicine, Los Angeles, CA 90024, [2]Department of Medicine, Southwestern Medical School, Dallas, TX 75235, [3]Department of Pathology, USC School of Medicine, Los Angeles, CA 90033, and [4]Department of Medicine, Pritzker School of Medicine, Chicago, IL. Carcinoma of the lung is associated with epidemiologic factors such as cigarette smoke and asbestos. While the mechanism by which these factors induce malignancy is not known, a growing body of evidence suggests that abnormalities in cellular regulatory genes such as transcription factors may be important in its pathogenesis. A group of immediate early transcription factors including *c-jun, c-fos* and EGR1 are known to bind to specific regulatory sequences and activate gene expression through a variety of cellular promoters. Since these genes are involved in regulating cellular growth properties we wished to determine whether the level of expression of these genes was altered in human lung tumors. Northern analysis was performed using *c-fos, c-jun* and EGR1 probes on RNA extracted from 103 pairs of lung tumor and adjacent normal tissue. This analysis revealed that 73% of the tumors as compared to adjacent normal tissue demonstrated markedly lower expression of these transcription factors. Moreover, the expression pattern appeared to be coordinate for all three genes in the majority of samples. This differential expression pattern was confirmed by immunohistochemical analysis using polyclonal antisera to the *c-jun, c-fos* and EGR1 gene products, which showed that only normal cells express these genes. Southern blot analysis demonstrated these genes to be unaltered and intact, thus indicating that underexpression did not result from their gross disruption. These data indicate that human lung tumors exhibited decreased expression of *c-fos, c-jun* and EGR1 as compared to their adjacent normal counterparts. Further studies of lung cell lines are currently underway to determine both the role and importance of decreased transcription factor gene expression, and how these changes affect the growth properties of malignant cells.

H 528 THE PKC-INDEPENDENT, PDGF-INDUCED c-fos mRNA EXPRESSION IN C3H/10T1/2 CELLS IS INACTIVATED IN THE 3-METHYLCHOLANTHRENE TRANSFORMED C3H/10T1/2 Cl 16 SUBCLONE

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The effect of platelet-derived growth factor (PDGF) on DNA synthesis and c-fos mRNA transcription was studied in the immortalized mouse embryo fibroblast C3H/10T1/2 Cl 8 (10T1/2) cells and the chemically transformed, tumorigenic subclone C3H/10T1/2 Cl 16 (Cl 16).

PDGF stimulated [^{3}H]thymidine incorporation 15-fold in the 10T1/2 cells in a dose-response manner approximately 15 hrs after initiation of treatment. PDGF had no effect on the DNA synthesis in the chemically transformed subclone. In the 10T1/2 as well as the Cl 16 cells, the dose dependent PDGF stimulation of c-fos mRNA synthesis was similar in both logarithmically growing and confluent cultures. Maximal induction of c-fos mRNA was at 30 minutes of PDGF treatment. c-fos mRNA was induced several fold by 12-O-tetradecanoylphorbol-13-acetate (TPA) in both 10T1/2 and Cl 16. Down-regulation of protein kinase C (PKC) activity by TPA pretreatment inhibited PDGF-stimulated c-fos expression in Cl 16 cells but did not affect this induction in the 10T1/2 cells. PDGF and TPA stimulated phosphatidylcholine synthesis (incorporation of ^{32}P) in both cell lines. PKC down-regulation completely inhibited the TPA effect while PDGF treatment still activated phosphatidylcholine synthesis 50%. Receptor binding experiments showed that the non-transformed and transformed cells had a comparable number of PDGF receptors, 5 x 10^5 and 1.8 x 10^5 receptors per cell respectively.

The present results provide evidence for an impaired mechanism of the PKC independent PDGF dependent c-fos signal transduction in the chemically transformed Cl 16 fibroblasts compared to nontransformed cells.

H 529 ALL-TRANS RETINOIC ACID (RA) REGULATES EXPRESSION OF MYELOBLASTIN (mbn) IN HUMAN PROMYELOCYTIC LEUKEMIA CELLS.

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Acute promyelocytic leukemia (APL) is characterized by a t(15;17) translocation, which fuses the newly described PML gene and the RA receptor α (RAR-α). RA induces differentiation and complete remission in patients with APL. Mbn, a serine protease involved in the control of growth and differentiation of human leukemic cells, is down-regulated during RA-induced differentiation of HL-60 cells. We found that mbn RNA was expressed in bone marrow mononuclear cells of patients with AML, including APL. In patients with APL, there was a dramatic up-regulation of mbn expression from as early as three days of RA treatment to the point of complete remission, where morphologically and cytogenetically normal bone marrow mononuclear cells continue to express mbn. In sera from patients with APL before and during RA treatment, Western blotting showed significant levels of secreted mbn. Since HL-60 cells do not have the t(15;17), we investigated (Labbaye et al, in preparation) RA-induced differentiation of NB4, a new cell line carrying the t(15:17) translocation (Lanotte et al, Blood **77**:1080). Regulation by RA of mbn RNA expression in NB4 cells differed in kinetics and pattern from that of HL-60 cells. In HL-60 cells, transcriptional down-regulation of mbn occured at two hours of RA-treatment, while, in NB4 cells, mbn was transcriptionally up-regulated at 16 hours. This up-regulation was followed by a decrease in mbn RNA to below baseline after 4 days of treatment. Thus, two RA-sensitive human leukemic cell lines differ in regulation of mbn by RA. The pattern of regulation in NB4 cells, which contain the PML/RAR-α fusion, more closely reflects the regulation of mbn in APL **in vivo**.

H 530 PROTEIN KINASE C-INDEPENDENT EXPRESSION OF STROMELYSIN BY PDGF, RAS ONCOGENE, AND PHOSPHATIDYLCHOLINE-HYDROLYZING PHOSPHOLIPASE C

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Changes in the expression of several genes play critical roles in cell growth and tumour transformation. A number of proteases are increased in some tumours, and the level of these enzymes correlate with the metastatic potential of several cancer cell lines. Stromelysin, with the widest substrate specificity, can degrade the extracellular matrix conferring metastatic potential to tumour cells. The mechanisms whereby growth factors and oncogenes control the expression of stromelysin are beginning to be characterized. In the study shown here we identify a region in the stromelysin promoter which is involved in the induction of stromelysin in response to platelet-derived growth factor (PDGF), PC-PLC, and ras oncogene. Our results are consistent with the notion that PDGF/PC-PLC induce stromelysin gene expression through a PMA/PKC-independent mechanisms by acting through elements in the stromelysin promoter distinct from the TPA-responsive element. Therefore, we demonstrate at a gene transcriptional level that the signalling mechanisms utilized by PDGF/PC-PLC differ from those triggered by PMA/PKC.

H 531 IDENTIFICATION OF DNA ELEMENTS REQUIRED FOR IL-6-INDUCED TRANSCRIPTIONAL ACTIVATION OF THE MOUSE *JUNB* GENE.

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IL-6 is a multifunctional cytokine regulating growth, differentiation and other cellular functions. IL-6 exerts its action through the specific IL-6 receptor composed of two chains, an 80KDa IL-6 binding chain and a signal transducing chain, gp130 (Hirano, Int. J. Cell Cloning 9:166,1991). The IL-6 signals activating two immediate early response genes, *junB* and TIS11, have been studied. Activation of *junB* and TIS11 transcription requires both a tyrosin kinase and an H7-sensitive kinase, which is distinct from PKC, PKA or Ca^{2+}/calmodulin-dependent kinases (Nakajima and Wall, Mol. Cell. Biol. 11:1409). To further elucidate the IL-6 signals and identify the DNA sequences essential for IL-6-regulated *junB* gene expression, we characterized the *junB* promoter and mapped a region involved in the rapid IL-6 induction of the *junB* gene in HepG2 cells. The IL-6 response element is located in the region of -149 to -124 bp containing two motifs, a c-ets-1 binding site (GCTTCCTG) and a CRE-like sequence (GTGACGCGA). Both of the two motifs were required for the full response to IL-6 since mutations of one of the two motifs markedly reduced IL-6 response and a CAT construct with three repeats of a DNA sequence of -163 to -139 bp containing only a c-ets-1 site did not respond to IL-6. Although forskolin (a PKA activator) alone showed much weaker activity for this DNA region, folskolin had synergistic effect with IL-6. Characterization of the DNA binding proteins acting on the DNA region is underway. The roles of the two motifs and the mechanisms of the synergy between PKA activity and IL-6 signals will be discussed.

H 532 HUMAN SERUM RESPONSE FACTOR (SRF) IS PHOSPHORYLATED BY THE DNA-ACTIVATED PROTEIN KINASE (DNA-PK), Sun-Yu Ng, Shu-Hui Liu, Jing-Tyan Ma, Shian-Huey Chiang, Susan P. Lees-Miller* and Carl W. Anderson*, Institute of Molecular Biology, Academia Sinica, NanKang, Taipei 11529, Taiwan, Republic of China and *Biology Department, Brookhaven National Laboratory, Upton, New York 11973, USA.

Transcriptional induction of the proto-oncogene c-*fos* and other Serum Response Element(SRE)-dependent cellular immediate early response genes by growth factors and other mitogenic signals is presumed to be mediated by the Serum Response Factor and its associated transcription factors. SRF is a 67-kDa phosphoprotein that binds the SRE as a dimer. SRF purified from HeLa cells has been tested as a substrate for several nuclear protein kinases. The most active kinase assayed was DNA-PK, a double-stranded DNA-activated serine/threonine kinase that also phosphorylates the transcription factor Sp1. SRF phosphorylation was stimulated significantly by SRE. DNA-PK recognizes serine or threonine residues followed by glutamine in SV40 T antigen and hsp90. Presumptive DNA-PK sites are present in the conserved C-terminal domain of SRF. The latter domain is both glutamine- and proline-rich and thus may function in transcriptional activation. A synthetic peptide harboring two -SQ- sites was phosphorylated by DNA-PK and also inhibited the DNA-PK phosphorylation of SRF polypeptides expressed in bacteria.

H 533 EXPRESSION AND REGULATION OF THE HUMAN CHOLECYSTOKININ GENE.
Karin Pedersen, Hans-Jürg Monstein, and Jens F. Rehfeld, Department of Clinical Chemistry, State University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark.

Cholecystokinin (CCK) belongs to the gastrin/CCK family of brain and gut peptide hormones. The CCK gene consists of 3 exons from which a messenger is transcribed. The messenger is translated into a prepropeptide of 115 amino acids. Posttranslational processing and finally α-amidation and tyrosin sulfation transforms the preproCCK into its bioactive form of which there are several. The longest bioactive CCK peptide found is 58 amino acids long and the shortest form is only 4 amino acids. All of the bioactive peptides share the same COOH-terminal containing the active site.

CCK was first found in the small intestine where it induces pancreatic enzyme secretion and gallbladder contraction. The CCK peptides found in the brain are thought to act as neurotransmitters. Furthermore, interesting studies of CCK in connection with schizophrenia, Huntington's disease, and eating disorders have been reported. As the cDNAs cloned form brain and gut in e.g. porcine tissues are identical, the different forms of the bioactive peptides are thought to be a consequence of tissue specific posttranslational events.

The CCK cDNA was cloned by Polymerase Chain Reaction from the human neuroepithelioma cell line SK-N-MC and the expression and posttranscriptional processing is being investigated in vitro and in vivo.

The promoter region of the CCK gene was cloned from the cell line mentioned above. The regulation of CCK gene expression is investigated by transient assays with different constructs of the promoter region for identification of binding sites of trans-acting factors.

H 534 THE EFFECT OF GROWTH HORMONE ON GROWTH AND GENE EXPRESSION IN INSULIN-PRODUCING CELLS. Elisabeth D. Petersen, Nils Billestrup and Jens H. Nielsen. Hagedorn Research Laboratory, Niels Steensens Vej 6, DK-2820 Gentofte, Denmark.

Growth hormone (GH) is a potent growth factor for the pancreatic ß-cell both in vitro and in vivo. Thus GH induces a 10-fold increase in mitogenic activity in monolayer cultures of newborn rat islets cells and a 3-fold increase in the incorporation of bromodeoxyuridine into the ß-cells of growth hormone releasing factor transgenic mice in vivo. A similar, but smaller, effect of GH was found in the rat insulinoma cell line RIN 5AH.

In order to elucidate the mechanism of action of GH on these cells, we analysed the effect of GH on the expression of the proto-oncogenes c-fos and c-jun as well as the insulin gene. By northern blot analysis little or no effect was found on the mRNA levels of c-fos and c-jun. In contrast, a 2-fold and a 3-fold increase in the insulin mRNA level was seen after 24 h and 48 h exposure to GH, respectively. A significant effect was seen already after 8 h.

Using a transient expression vector containing the rat insulin-1 promoter with the CAT gene as reporter gene in transfected RIN 5AH cells, GH induced a 2-fold increase in the CAT activity, whereas no effect was found when the c-fos promoter was used.

These results indicate, that the growth promoting effect of GH in the RIN 5AH cells does not involve induction of c-fos and c-jun. It may be speculated, that the stimulation of the expression of the insulin gene may contribute to an autocrine growth promoting effect of insulin in these cells.

H 535 ISOLATION AND CHARACTERIZATION OF THE MURINE C-FGR GENOMIC LOCUS CONTAINING THE CODING AND REGULATORY REGIONS, Nitaya Podhipleux and Cheryl L.Willman. Depts. of Cell Biology and Pathology, Univ. of New Mexico School of Medicine, Albuquerque, NM 87131.

The c-fgr proto-oncogene is a member of the src family of non-receptor tyrosine kinases. C-fgr is expressed exclusively in myeloid cells where it may play a role in the control of proliferation and functional activation in mature monocytes as well as in the development of the monocytic lineage from hematopoietic progenitor cells (Willman et al., PNAS 86:4254-58, 1987). It has been shown in our laboratory that the c-fgr cDNA encodes two proteins of 53 and 59kd through usage of alternative translation initiation codons in the second exon. These two proteins, one containing and one essentially lacking the amino terminal domain have different subcellular locations and functions in normal monocytic cells. Despite this knowledge, the murine c-fgr genomic locus, the promoter, and its regulatory elements have yet to be identified and characterized. In order to isolate and characterize the c-fgr genomic locus and upstream regulatory elements, a lambda GEM-11 murine liver genomic DNA library was screened with a mcfgr53 probe (Yi and Willman, Oncogene 4:1081-87, 1989). Three overlapping phage clones with a combined length of 33kb were obtained. These overlapping clones contain the entire c-fgr coding exons plus 7 kb of sequence 5' to the c-fgr translation initiation site. The entire locus had been subcloned and a detailed restriction enzyme map has been obtained. Using anchored PCR, the 5' untranslated region of the c-fgr cDNA has been confirmed and extended. This region is encoded by at least two exons upstream of exon "2", which contains the translation initiation site. These exons have been partially mapped and the intron-exon structure of the amino terminal exons are being determined. The transcriptional initiation site(s), promoter and regulatory regions are under further investigation and will be tested in heterologous systems for functional activity.

H 536 USF DNA BINDING AND TRANSCRIPTIONAL POTENTIALS ARE REGULATED IN A REDOX DEPENDENT MANNER.

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USF is a transcription factor that binds to the core sequence CACGTG. Purified from HeLa cells, it consists of two unrelated polypeptides of 43 and 44 kDa. The 43 kDa protein contains a basic/helix-loop-helix/leucine zipper domain (B/HLH/LZ), and is indistinguishable from the natural 43/44 kDa USF in its ability to bind to DNA and to activate transcription through its cognate DNA target. We show here that the DNA binding of USF^{43} is strongly dependent upon redox conditions, suggesting that the only two cysteine residues presents in USF^{43}, both found within the B/HLH/LZ domain, are targets of this regulation. Site directed mutagenesis studies demonstrate that each of these cysteine residues is sufficient to confer redox dependent regulation, indicating that intermolecular disulfide bridges are involved. This is confirmed by the observation of disulfide linked multimeric complexes under non-reducing conditions. This suggests that covalently linked USF dimers or polymers have a reduced affinity for DNA, probably because of some structural conformational locking created by the disulfide bridges. In agreement with this model, USF^{43}SS, in which both cysteines have been changed to serine, does not form covalently crosslinked dimers under non-reducing conditions, but is still present as dimers. Furthermore, its DNA binding potential is unaffected by redox conditions. Finally, *in vitro* transcription experiments demonstrate that the transcriptional potential of USF^{43} is dependent upon redox conditions, whereas that of USF^{43}SS is not.

Taken together, these data suggest that USF transcriptional potential may be regulated in the cell according to redox changes. Similar types of regulations have been reported for Fos/Jun (1) and NFkB (2), indicating that redox conditions may be a general way of regulating the activity of certain transcription factors *in vivo*.

(1) Abate et al, Science (1990) **249**, pp 1157-1160.
(2) Schreck et al, EMBO J. (1991) **10**, pp 2247-2258.

H 537 TRANSCRIPTIONAL ACTIVITY OF THE EFII ENHANCER ELEMENT IS ACTIVATED BY *v-src* AND *c-myc* CELL TRANSFORMATION, BUT REPRESSED BY *v-myc* TRANSFORMATION. Rosalie Sears and Linda Sealy, Dept. of Cell Biology, Vanderbilt University, Nashville, TN. We have characterized three protein complexes in chick embryo fibroblasts, called EFIIa, EFIIb and EFIIc, which bind to a 38bp sequence at the 5' end of the RSV LTR enhancer extending from nucleotides -229 to -192, which we refer to as the EFII cis element (Sears and Sealy, in preparation). We have shown that six copies of the EFII cis element linked to a CAT reporter gene ($EFII_6$-CAT), activate transcription 40-fold when transfected into CEF. The avian leukosis virus is known to have transduced a number of cellular oncogenes, including *src and myc, to produce the acutely* transforming strains RSV and MC29, respectively. Therefore, we wished to ask whether src or myc oncoproteins would affect the transcriptional activity of the EFII cis element, and/or the binding activity of EFIIa, b or c to this cis element. We found a positive correlation between increased transcriptional activity using $EFII_6$-CAT (2-3 fold induction) *in vivo*, and increased binding activity (2-3 fold) of EFIIa, b and c *in vitro*, when normal CEF were compared with CEF transformed by RSV. Interestingly, when we performed the same experiments with CEF infected by the MC29 virus (expressing a 110kd gag-myc fusion protein) we observed a 3-4 fold decrease in transcriptional activity from $EFII_6$-CAT, despite a 4-5 fold increase in EFIIa, b and c binding activity *in vitro*. Further analysis using a quail embryo fibroblast cell line transformed by MC29 (HB1) also demonstrated a 3-4 fold decrease in transcription from the EFII cis element, when compared with a chemically transformed quail fibroblast cell line, QT-6. The repression of transcription was found to be specific for the EFII enhancer element since all other constructs tested, including a USF driven reporter construct, did not show a significant decrease in transcriptional activity in the MC29 transformed cell lines. We have also generated stable clones transfected with a CMV driven *c-myc* construct using the QT-6 cell line. Analysis of these *c-myc* transformed clones demonstrated a 2-3 fold increase in transcription from the EFII cis element specifically. Our data suggests that cell transformation by *v-src* or *c-myc* leads to an increase in transcription from the EFII enhancer element, while transformation with a gag-myc fusion protein, which still results in increased EFIIa, b and c binding activity, represses transcription from the EFII DNA sequence. Preliminary evidence indicates that the inhibition of EFII driven transcription in MC29 infected cells may be due to the gag portion of v-myc.

H 538 THE HIP1 SITE IS REQUIRED FOR GROWTH REGULATION OF THE DHFR PROMOTER, Jill Slansky and Peggy Farnham, Department of Oncology, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI, 53706

The transcription rate of the DHFR gene increases at the G1/S boundary of the proliferative cell cycle. Through analysis of transiently and stably transfected NIH 3T3 cells, we have now demonstrated that DHFR promoter sequences extending from -270 to +20 are sufficient to confer similar regulation on a reporter gene. We observe a 13 fold increase 12 hours after release of serum starvation. Mutation of a protein binding site that spans the sequences for -16 to +11 in the DHFR promoter did not effect basal activity of the promoter, although it resulted in loss of the transcriptional increase at the G1/S boundary. Currently we are examining the effect of point mutations in this region on growth regulation. Purification of an activity from HeLa nuclear extract that binds to this region enriched for a 180 kDa polypeptide (HIP1). We are now determining if binding to the HIP1 site changes in stage specific NIH 3T3 extracts.

H 539 ACTIVITY OF THE HUMAN ENKEPHALIN ENHANCER IS MODULATED BY THE FORMATION OF HAIRPIN STRUCTURES THAT ARE RECOGNIZED BY ENHANCER BINDING PROTEINS. Craig Spiro and Cynthia T. McMurray, Departments of Pharmacology and Biochemistry and Molecular Biology, Mayo Foundation, Rochester, Mn. 55905.
Proenkephalin, the product of the human enkephalin gene, is processed to peptides that function both as neurotransmitters and as neurohormones. Enkephalin expression can be activated via G-coupled receptors, growth factor receptors, steroid hormones, and ligand-gated ion channels. The expression of the gene is tightly regulated, at least in part by an enhancer element that responds to both cyclic AMP and phorbol esters. In vivo and in vitro data suggest that the enhancer undergoes a duplex to cruciform conformational change upon cAMP activation and that the cruciform state represents the induced conformation of the gene. We have assigned the structure of both the GT and the AC hairpin arms using ^{1}H-NMR. Each arm forms with a 10bp stem and a 3bp loop and each contains two mismatched base pairs. In vitro, we have found that the enkephalin enhancer duplex will completely convert into two stable hairpin structures. We have also found that mutations which potentially stabilize a hairpin structure within the enhancer have a positive effect on expression in vivo while mutations which potentially destabilize a hairpin structure abolish cAMP induction. One feature of the hairpin formation is that double-stranded sites for protein binding are retained within the arms of the hairpin structures. Three proteins binding sites, AP1, AP2 and CREB, are found within the enkephalin enhancer region. Each of these proteins has been implicated in the cAMP response for other genes. Cotransfection of the human enkephalin enhancer with either CREB or AP1 results in significant increase in the transcription of the enkephalin gene over basal values in several cell lines. Using a gel shift assay, we have found that the two potential CRE-binding proteins, CREB protein and the AP1 complex, specifically recognize and bind both to the duplex form of the enkephalin enhancer and to the GT hairpin. The GT hairpin forms from the top strand of the duplex enhancer and contains two GT mismatched pairs. Neither protein recognizes the AC hairpin with significant affinity. The AC hairpin forms from the bottom strand of the enkephalin duplex and contains two AC mismatched pairs. Comparison of the binding for purified CREB to the enkephalin duplex and to the GT hairpin indicates that the CREB protein binds to the GT hairpin with a 5-fold greater affinity. Binding to both the duplex and to the GT hairpin is abolished by competition with an oligonucleotide containing a consensus CREB binidng site. Purified CREB binding to the GT hairpin is strong and occurs with equal affinity as to an oligonucleotide containing the somatostatin consensus CREB site. Additionally, purified CREB does not bind to GT hairpins that contain point mutations in either the T residue or the G residue within the conserved "CGTCA" portion of the CREB binding site. Under identical conditions, purified CREB does not bind to an oligonucleotide containing an AP2 site and binds with a detectable but lower affinity to an oligonucleotide containing an AP1 consensus site.

H 540 CLONING OF cDNAs REGULATED BY C-MYC, Sean V. Tavtigian and Barbara J. Wold, Division of Biology 156-29, California Institute of Technology, Pasadena, CA 91125.

A number of immediate early gene products, such as c-fos, c-jun, and c-myc, are transcription factors with oncogenic potential. In the context of the cell cycle, identification of their regulatory targets is an important step towards understanding their function. We have used a two step cloning protocol to identify genes regulated by c-myc. Hypothesizing that the activity of c-myc is responsible for regulating an identifiable subset of the genes induced or repressed in mid G1, we prepared libraries from both growth arrested and mid G1 cells, differentially screened those libraries with corresponding subtracted cDNA probes, and obtained a panel of cDNA clones subject to the expected temporal response to serum stimulation. By examining the expression of these cDNAs in cell lines that conditionally overproduce c-myc, we were then able to select a subset whose regulation depends, at least in part, on its activity.

To date, it appears that about one quarter of the mRNAs subject to mid G1 regulation by serum respond in similar fashion to c-myc overproduction. While there is no *a priori* way of knowing how many regulatory steps occur between c-myc overproduction and the observed accumulation or decay of a particular transcript, we have isolated a large enough number of "c-myc regulated" clones to begin dividing them into groups subject to direct vs. indirect and transcriptional vs. post transcriptional regulation. We are also examining their expression in cell lines which inducibly co-express c-myc with its pairing partner max.

H 541 TESTIS C-MOS PROMOTER REGULATION BY A MALE GERM CELL-SPECIFIC TRANSCRIPTION FACTOR, F.A. van der Hoorn, H.A. Tarnasky & N. Higgy, Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada T2N 4N1.

The c-mos gene, which is predominantly expressed in germ cells, encodes a serine-threonine protein kinase, which is involved in regulation of meiosis during oogenesis and which may be a component of Cytostatic Factor. We recently detected 3 c-mos RNAs (5, 3.6 and 1.7 kb) in rat testis: however, $p43^{c-mos}$ is only found before meiosis and is postulated to play a role similar to that in oogenesis. To gain insight into the transcription regulation of c-mos in the testis, we identified the 1.7 kb c-mos RNA cap site using RACE PCR. The promoter, as defined by this cap site, is 0.55 kb upstream of the coding region, has a TATA-like box and is predicted to generate a 1.65 kb c-mos RNA contiguous with genomic sequences. The promoter behaves as a testis-specific, weak promoter in in vitro transcriptions using rat seminiferous tubule- and liver nuclear extracts. It binds a testis-specific nuclear factor at -121, the sequence of which is homologous to the binding site for a testis-specific transcription factor TTF-D, which we recently characterized in the promoter of another male germ cell-specific gene RT7. We also show that the c-mos enhancer, which is located 1.5 kb upstream of the testis c-mos promoter, only binds ubiquitous factors in male germ cells. One of these is a novel 61 kD positive transcription factor, that we call TF-ME, distantly related to OTF as defined in gel retardation experiments.We propose that c-mos expression in various tissues (testis, ovary, muscle) is regulated primarily by -differently located- tissue-specific promoters. The role of the enhancer may be to increase the activity of these different promoters.

H 542 MAX STIMULATION OF MYC DEPENDENT TRASCRIPTION; IS IT INCREASED DNA BINDING OR INCREASED ACTIVATION POTENTIAL? Antonis S. Zervos and Roger Brent. Department of Genetics, Harvard Medical School and Department of Molecular Biology Massachusetts General Hospital, Boston MA 02114

We have used a novel system of LexA fusion proteins to study the Myc oncoprotein and its interacting partner Max. In these experiments, we used wild-type and altered specificity LexA DNA binding domains to cause heterodimers of human Myc and Max to form on a mixed specificity operator. Our results showed that Myc alone is a weak activator while Max alone is transcriptionally inert. Unexpectedly however, we found that heterodimers of Myc and Max activated transcription more strongly than either protein alone. Native Max stimulated transription of a number of Myc fusion proteins that contain different binding moeities. This stimulation required the C-terminal oligomerization motifs for the physical interaction and the N-terminus and middle of protein for the enhanced activation. Further experiments suggested that interaction with Max helps Myc stimulate transcription by making it into a more potent activator, perhaps by contributing an activating surface that is only functional when juxtaposed to Myc. It is possible that stimulation of Myc transcription by Max is important for Myc-dependent oncogenesis.

H 543 pAT 133: A NOVEL MEMBER OF A CLASS OF DISTINCT GROWTH FACTOR-INDUCED GENES WITH ALMOST IDENTICAL ZINC-FINGER DOMAINS, Peter F. Zipfel, Corinna Holst, Hans-Joachim Müller, Alexandra Bialonski and Chistine Skerka, Bernhard Nocht Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, D-2000 Hamburg 36, Germany.

The mRNA of pAT 133 is induced in T lymphocytes upon transition from a resting state (G_0) through the early phase of the cell cycle (G_1). Primary structure analysis shows that the encoded protein contains three tandem zinc finger sequences of the type Cys_2-X-His_2. This zinc finger region, which binds DNA in a sequence specific manner, has a high degree of similarity (over 80% on the amino acid level) with three previously described transcription factors pAT 225/EGR1, pAT 591/EGR2 and EGR3, and a lesser homology to the candidate Wilms tumor gene WT33 and to transciption factor SP1. Except for the conserved zinc finger domains, the amino acid sequences of the four highly related proteins are distinct. This structural similarity suggests, that the pAT 133 gene encodes a transcription factor with a unique biological function.

Comparing the regulation of the related zinc finger encoding genes pAT 225/EGR1, pAT 591/EGR2 and pAT 133 shows coordinate induction upon mitogenic stimulation of resting T lymphocytes and of resting fibroblasts. However, upon transition from a proliferating (G_1) to a resting state of the cell cycle these genes are differently regulated, suggesting distinct biological roles of the proteins in the control of cell proliferation. In human histiocytic U937 cells mRNA of pAT 133 is constitutively expressed, while mRNA of pAT 225/EGR1 is induced upon induction of terminal differentiation. In contrast mRNA representing pAT 591/EGR2 can not be detected in these cells.

The human pAT 133 gene is organized in two exons, which are separated by one intron. The regulatory region shows a TATA-box and several regulatory elements (e.g. serum response elements) which seem to regulate the transcriptional induction of the pAT 133 gene.

Late Abstracts

IDENTIFICATION OF A NEW (6;12) TRANSLOCATION IN MOUSE PLASMACYTOMA

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Mouse plasmacytomas (MPCs) reguarly carry a chromosomal translocation that leads to the juxtaposition of c-myc to one of the three Ig loci. The translocation is belived to act by constitutive activation of c-myc.

We have recently found a MPC carrying a new, previously non-identified, translocation. It did not have the usual Ig/c-myc translocation, but contained a 6;12 translocation instead. The breakpoint on chromosome 6 corresponds to the bands B3/C1, the region of the Ig kappa gene. The chromosome 12 breakpoint has been mapped to 12(B1-C1), which corresponds to the localization of N-myc.

Northern analysis showed that the tumor expresses high levels of N-myc, while no c-myc expression was detected. Molecular evidence will be presented which supports the concept that this tumor represents the first example of N-myc activation by chromosomal translocation.

UV-INDUCED SECRETED PROTEINS AND DECREASE IN REPLICATION FIDELITY

Jan J.B. Boesen, Nicole Dieteren, Elise Bal, Paul H.M. Lohman, and Jo W.I.M. Simons, MGC-Department of Radiation Genetics and Chemical Mutagenesis, State University of leiden, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands.

UV light results besides the induction of DNA-damage also in the induction of signal transduction pathways. Part of the 'UV response' forms the secretion of 'extracellular-protein-synthesis-inducing-factors' (EPIF) which is able to turn on part of the very same response in non-irradiated cells[1]. EPIF has been identified as basic Fibroblast Growth Factor (bFGF), Interleukin-1 (IL-1) (or immunologically related proteins), Tumor Necrosis Factor-α (TNF-α) and several unidentified proteins[2]. There are indications that induction of signal induction pathways leads to genetic instability[3]. An assay is in development, using a mouse T-lymphoma cell line, to assess the induction of genetic instability by EPIF and EPIF-components. It was found that the spontaneous mutation rate at the *hprt*-locus varies 200-fold depending on the culture conditions. The induction of secreted proteins is determined by SDS-PAAGE. EPIF leads under defined culture conditions to an enhanced mutation rate. EPIF may be the mechanism by which cells propagate information from a mutagen-hit cell to other non-treated cells. One of the effects of EPIF may be the decrease of replication fidelity in these cells.

[1]Schorpp et al. (1984) Cell *37*, 861-868.
[2]Stein et al. (1989) J. Virology *63*, 4540-4544.
[3]Boesen et al. (1991) Carcinogenesis *12*, 487-492.

Transmembrane signalling potential of antigen receptors on pre-B and mature B lymphocytes.

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Throughout B cell development, different types of antigen receptors are expressed. On pre-B cells, that contain μ heavy (H) chain protein, but have not yet productively rearranged the light (L) chain locus, small amounts of μ chains are found at the cell surface in association with the φ L chain complex. This pre-B cell receptor may play a role in regulation of Ig gene rearrangement. Early B cells express transmembrane (m)IgM, i.e. a complex of μ and L chains, while mature B cells coexpress mIgM and mIgD, or may undergo class switching to express mIgG, mIgA or mIgE. Antigen binding to mIg can lead to either B cell activation, proliferation and differentiation into antibody secreting plasma cells or to clonal anergy or cell death. A structural basis for the differential signalling potential of antigen receptors depending on the B cell differentiation stage may be found in the receptor itself and/or in the enzymatic machinery coupled to the receptor. We have investigated the composition of the pre-B cell and mature B cell receptors and found that in both cases, they are associated with disulfide-linked protein products of the *mb-1* and *B29* genes. It is not yet clear whether these two proteins are identical in all complexes, or structurally highly related. In addition, tyrosine kinase and serine kinase activities are associated with these receptor systems, that have the Ig-linked *mb-1/B29* components, but not the Ig H chains, as substrates. These findings open up the possibility that phosphorylation of the Ig-linked molecules is a requirement for coupling of the antigen receptor complexes to phospholipase Cγ and perhaps other enzymes initiating signalling cascades.

FUNCTIONAL LINK BETWEEN RAS AND RAF IN ONCOGENIC TRANSFORMATION,

Antonio Cuadrado, Instituto de Investigaciones Biomédicas (CSIC) Madrid 28029, Spain.

The purpose of this study was to investigate the possible connection between the protein products of H-ras and c-raf-1 genes in the same signaling pathway. The NIH 3T3 fibroblast cell line C41-22, which contains the oncogene H-ras under the control of a glucocorticoid inducible promoter, was used to determine the temporal relationship between oncogenic H-ras p21 appearance and activation of RAF-1 kinase. Such activation occurred simultaneously with the expression of H-ras p21 (2-4 hours of dexamethasone treatment) and long before phenotypic transformation could be observed (about 48 hours of treatment). RAF-1 kinase activation occurred in a protein kinase C-independent manner since ras transformed cells depleted of PKC by chronic phorbol ester treatment still exhibited RAF-1 kinase activation and phosphorylation. The effect of RAF-1 kinase overexpression on the transforming response to ras oncogenes was also analyzed. 30 fold overexpression of RAF-1 kinase did not confer the malignant phenotype to transfected NIH 3T3 cells. However, overexpression of RAF-1 kinase made cells markedly more sensitive to transformation by both normal and oncogenic H-ras p21. Those transformed cells containing high levels of raf p74 protein consistently exhibited a lower level of the transfected H-ras p21, suggesting that overexpression of raf p74 lowers the threshold levels of ras necessary to induce transformation. It is proposed that RAF-1 kinase is at least in part responsible for some of the PKC-independent activities of H-ras p21, in particular, morphologic transformation.

DNA POLYMORPHIC DIFFERENCES BETWEEN NORMAL AND TUMOR SAMPLES CAN BE DETECTED USING POLYMERASE CHAIN REACTION (PCR) WITH ARBITRARY PRIMERS, Peter Economou[1], Gregory Kelly, Jonathan Samet[1] and John F. Lechner, Inhalation Toxicology Research Institute, Albuquerque, NM 87185. [1]Department of Pulmonary and Critical Care Medicine, University of New Mexico at Albuquerque, NM 87131.

Williams, et al., NAR 18:6531('90) and Welsh, et al., NAR 19:303('91) have reported that simple and reproducible fingerprints of complex genomes can be obtained using single arbitrary primer PCR (AP-PCR). We have found that the AP-PCR technique can also be used to detect polymorphic differences between normal and tumor DNA samples from human donors with lung cancer. Specifically amplified DNA sequences are obtained when the PCR protocol consists of first, two low stringency cycles of 94°C for 5 minutes, 48°C for 5 minutes and 72°C for 5 minutes and second, 30 standard high stringency cycles. The arbitrary primers used to date have been the 5' lambda GT10 and 3' lambda GT10 cloning sequences of 21 and 24 nucleotides in length, respectively and AP2, a 10 nucleotide sequence of CGGCCCCGGC. These primers yield distinctive fingerprints consisting of more than 50 bands. Polymorphic differences between normal and tumor DNAs have been found with all primers. In addition, donor specific polymorphisms have also been detected. The results of these preliminary observations suggest that AP-PCR may be a useful technique to reveal polymorphisms between human normal and tumor tissues that may be seminal in transformation. AP-PCR may also be useful in revealing animal tumor/normal polymorphisms where restriction fragment length polymorphism analysis is impractical.

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14-HYDROXY-RETRORETINOL, A CELLULAR DERIVATIVE OF RETINOL ACTS HIGHLY SYNERGISTICALLY WITH GROWTH FACTORS IN INDUCING THE IMMEDIATE EARLY GENE c-fos, Thomas M. Eppinger, Genshu Tate, Jochen Buck and Ulrich Hämmerling, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

14-Hydroxy-retro retinol (14-HRR), a cellular derivative of vitamin A, has recently been identified and characterized in our laboratory. It has growth-promoting effects on a variety of cell lines. In an attempt to find the mode of action of this newly described retinoid we stimulated density-arrested, quiescent murine fibroblasts with 14-HRR and growth factors known to induce immediate early gene expression. While 14-HRR alone did not show an effect in this assay, it exhibited a highly synergistic effect on c-fos induction when used in combination with PDGF. 14-HRR may be part of a new intracellular activation pathway or provide a link between the known pathways of kinase receptors and gene activation.

Loss of heterozygosity for alleles on chromosome 11 in Neuroblastoma: Correlation with advanced stage tumors. Eri S.Srivatsan, C. Patrick Reynolds and Robert C.Seeger. Division of Hematology/Oncology, Children's Hospital of Los Angeles, Department of Pediatrics, USC school of Medicine, 4650 Sunset Blvd, Los Angeles, CA-90027

Molecular genetic studies performed previously on 15 Neuroblastomas and their corresponding constitutional cells had shown loss of heterozygosity for alleles on chromosome 11 in five (33%) of the tumor samples (Srivatsan et.al., Prog.Clin.Biol.Res 377:91-98, 1991). In order to better determine the deletion frequency of chromosome 11 sequences, RFLP analysis was carried out on forty five different primary tumors and five cell lines. Deletion of chromosome 11 sequences was observed in 13 of 42 (31%) informative cases (38 tumors and 4 cell lines). Both 11p and 11q probes were informative in 8 tumors: Loss of all of chromosome 11, of only 11p sequences, and of 11q sequences was observed in 4, 1 and 3 tumors respectively. Thus 11q may be lost more often than 11p. Also one of the primary tumors contained a cytogenetic translocation involving q12 region of chromosome 11. Chromosome 11 deletions were mostly restricted to advanced stage tumors, which suggests that the sequences localized to chromosome 11, particularly those of 11q might play a role in the progression of neuroblastomas.

Differential substrate specificity of phosphotyrosine phosphatases,

Reiner Lammers[1], Susanne Müller[1], Deborah Cool[2], Ronald Herbst[1], Nicholas Tonks[3], and Axel Ullrich[1,1] Dept. of Molecular Biology, Max-Planck-Institut für Biochemie, 8033 Martinsried, FRG, [2] Dep. of Biochemistry, University of Washington, Seattle, WA 98195 USA,[3] Cold Spring Harbor Labs, Cold Spring Harbor, NY 11724 USA

The specificity of interaction of phosphotyrosine-specific phosphatases (PTP) with receptor tyrosine kinases was investigated in a model system that allows overexpression of different proteins in one cell. Cytosolic PTPs T-cell phosphatase and phosphatase 1b and transmembrane PTP CD45 were coexpressed with different receptor tyrosine kinases (RTK) of subclass III and the phosphotyrosine content of receptor molecules was determined. While coexpression of CD45 reduced phosphotyrosine content in PDGF-Rs and CSF-1-R by about 50% phosphotyrosine in the Kit/SCF-R was reduced to less than 5%. The cytosolic PTPs reduced phosphotyrosine in PDGF-R to about 10% of control while for KIT- and CSF-1-R the effect was similar to that of CD45. All PTP were also tested with RTK of subclasses I and II. These receptors behaved similar as the CSF-1-R in their susceptability to PTP action. The decrease in phosphotyrosine content was time dependent and there appears to be no preference for specific tyrosine phosphate residues within a receptor molecule. Additional expression of an RTK substrate can inhibit the dephosphorylating effect of the phosphatase. Interestingly ligand-induced degradation of activated receptors is not prevented by high level coexpression of a phosphatase. The effect of PTP expression on cell transformation by RTK is presently under investigation.

PRODUCTION OF SPECIFIC ANTIBODIES TO THE 70K MITOGEN-ACTIVATED S6 KINASE: IDENTIFICATION OF AN ANALOGOUS KINASE IN *XENOPUS LAEVIS* OOCYTES, Heidi A. Lane, Simon J. Morley, Marcel Dorée, Sara C. Kozma and George Thomas, Friedrich Miescher Institute, 4002 Basel, Switzerland, CNRS, Montpellier, France

We have purified the mitogen-activated 70K S6 kinase from vanadate-treated 3T3 cells (1) and, on a larger scale, from cycloheximide-stimulated rat liver (2,3). Using protein sequence data derived from the rat liver S6 kinase, the enzyme was cloned and shown to be a ser/thr kinase belonging to the second messenger family of protein kinases (4). The biochemical basis of cell cycle control has been extensively studied in *Xenopus* oocytes undergoing meiotic maturation in response to hormonal stimuli. Previously, only one family of S6 kinases has been identified in this system (M_r 90,000), which are activated during maturation (5), and are distinct from our enzyme. Based on the cDNA sequence of the 70K S6 kinase we have been able to demonstrate by PCR that the mRNA for this kinase is also present in stage VI oocytes. Using specific polyclonal antibodies raised against synthetic peptides, we have also been able to demonstrate that the protein is expressed and has activity in oocytes and that following progesterone treatment the basal activity is increased 10-fold within 1 hr with kinetics distinct from those of the 90K S6 kinase family. Data will be presented on the further characterisation of the role of the 70K S6 kinase during *Xenopus* oocyte maturation.

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2. Kozma, S.C. et al. (1989) EMBO J. **8**:4125
3. Lane, H.A. & Thomas, G. (1991) Meth. Enzym. **200**:268
4. Kozma, S.C. et al. (1990) PNAS **87**:7365
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INDUCTION OF NF-κB-LIKE ACTIVITY BY PLATELET-DERIVED GROWTH FACTOR IN MOUSE FIBROBLASTS. Nancy E. Olashaw and W.J. Pledger, Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232

Nuclear factor κB (NF-κB) modulates the expression of numerous genes via interaction with a specific DNA sequence termed the κB site. Activation of NF-κB occurs at a post-translational level, in response to a variety of agents and in a variety of cell types, most notably B and T lymphocytes. We have found that an activity that binds specifically to the κB site is present in density-arrested Balb/c-3T3 fibroblasts and is induced by platelet-derived growth factor (PDGF), a potent mitogen for these cells. Increased κB binding activity, as evaluated by electrophoretic mobility shift assays, was observed in nuclear extracts of cells treated for 1-4 h (but not 15 min) with the BB isoform of PDGF. The activity induced by PDGF exhibited properties charcteristic of those previously described for NFκB including inducibility by deoxycholate and cycloheximide. 12-O-tetradecanoylphorbol 13-acetate (TPA), the AA isoform of PDGF and vanadate also stimulated κB binding activity whereas insulin-like growth factor I and transforming growth factor β were ineffective. Both PDGF isoforms, but not TPA, induced this response in cells depleted of protein kinase C. These studies suggest that NF-κB-like factors may participate in the expression of PDGF-inducible genes.

TRANSFORMATION BY THE BCR-ABL ONCOGENE REQUIRES THE PRESENCE OF BCR SEQUENCES WHICH BIND TO THE ABL SH2 REGULATORY DOMAIN AS WELL AS TO SH2 DOMAINS PRESENT IN A SUBSET OF SIGNALLING PROTEINS. Ann Marie Pendergast,[1] Alexander J. Muller,[1] Marie H. Havlik[2] and Owen N. Witte[1,2]. [1] Department of Microbiology and Molecular Genetics and Molecular Biology Institute. [2] Howard Hughes Medical Institute, University of California, Los Angeles, CA 90024.

BCR-ABL is a chimeric oncogene implicated in the pathogenesis of Philadelphia chromosome-positive human leukemias. BCR sequences specifically activate the tyrosine kinase and oncogenic potential of ABL. We have shown that BCR sequences in the BCR-ABL chimera bind specifically to the SH2 domain of ABL. These BCR sequences are essential for BCR-ABL-mediated transformation. The binding of BCR to the ABL SH2 domain represents a new class of SH2-protein interactions which requires the presence of phosphoserine/phosphothreonine but not phosphotyrosine residues on BCR.

While phosphotyrosine-containing proteins bind rather uniformly to isolated SH2 domains, the phosphoserine-/phosphothreonine-containing BCR sequences bind to only a subset of those SH2 domains. These results indicate that the specificity of binding to SH2 domains of phosphoserine-/phosphothreonine-containing proteins is different from that of phosphotyrosine-containing proteins. These findings suggest a role for BCR in cellular signalling pathways and in the activation of the BCR-ABL oncogene through a novel protein-SH2 domain interaction.

DEREGULATED HOMEOBOX GENE EXPRESSION CONTRIBUTES TO LEUKEMIA BY IMPEDING DIFFERENTIATION. A. C. Perkins*, J. M. Adams, S. Cory. Walter and Eliza Hall Institute of Medical Research, P.O. Box Royal Melbourne Hospital, Parkville, VIC., AUSTRALIA 3050.

The murine myelomonocytic leukemia, WEHI 3B, is characterized by aberrant expression of genes for the growth factor interleukin 3 (IL-3) and a homeobox gene, Hox 2.4, due to insertion of endogenous retroviral-like elements into the 5' regulatory regions of both genes. In order to unravel the relative contributions of autocrine growth factor production and ectopic expression of the homeobox gene to the oncogenic process, we have delivered the genes either simultaneously or separately into murine bone marrow cells via recombinant retroviral vectors. As reported by others, enforced expression of IL-3 alone elicited a fatal but non-transplantable myeloproliferative syndrome in mice reconstituted with infected cells. In contrast, mice reconstituted with cells expressing both Hox 2.4 and IL-3 developed a transplantable blast cell disease that was transplantable (1). Mice reconstituted with marrow expressing Hox 2.4 alone showed no clinically apparent disease, although excess circulating granulocytes and platelets were present and excess progenitor cells were demonstrable in the marrows and spleens.

The marrow from mice reconstituted with Hox 2.4/IL-3 expressing cells yielded many clonally distinct compact colonies in agar that were readily expanded into immortalized myelomonocytic lines. The results indicated that IL-3 and Hox2.4 are a potent synergistic combination and may in fact suffice for full transformation of myeloid precursors. More recently, non-tumorigenic, factor-dependent cell lines of various lineages and stages of differentiation have been generated from marrow expressing Hox 2.4 alone. These lines readily differentiate when removed from high concentrations of Il-3 and exposed to the appropriate growth factors or stromal environment. A subset of lines displayed certain characteristics of primitive stem cells: cobblestone formation on stromal cell lines and day 13 spleen colony forming unit (CFUs) activity. These lines provide a useful tool to analyse the molecular events that accompany lineage commitment and differentiation of haematopoietic stem cells.

This work directly demonstrates that unscheduled expression of a homeobox gene can impede differentiation of hematopoietic progenitors and collaborate with the growth factor gene, IL-3, to transform normal hematopoietic cells into immortalized, transplantable cells.

1. Homeobox gene expression plus autocrine growth factor production elicits myeloid leukemia. Perkins, A.C., et al. *PNAS* 87; 8398-8402.

IDENTIFICATION OF NOVEL INTRACELLULAR POLYPEPTIDES AT 55 AND 72KD ASSOCIATED WITH THE TCR/CD3 COMPLEX, Christopher E. Rudd, Craig H. Zalvan, Masahiro Yamamoto and Antonio J. da Silva Division of Tumor Immunology, Dana-Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, MA 02115

Engagement of the T cell antigen-receptor complex (TcR/CD3) induces the rapid tyrosine phosphorylation of a spectrum of substrates whose modification appears to be crucial to the activation process. Two protein tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ which associate with the CD4/CD8 and the TcR/CD3 complex, respectively, could mediate this cascade. Here, we reveal that the TcR/CD3 complex co-precipitates two other novel polypeptides at 55Kd (p55) and 72Kd (p72) in addition to $p59^{fyn}$ (at 59kd and a lower Mr species at 57Kd). Peptide mapping and re-precipitation analysis showed p55 to be distinct from CD4 associated $p56^{lck}$. Purified p55 is labeled exclusively on tyrosine residues and can be detected in direct association with $p59^{fyn}$. Significantly, in vitro phospholabelling allowed detection of p55 only in T cells. TcR/CD3 complex also co-precipitated a prominent band at 72Kd which is comprised of an altered form of fyn ($p72^{fyn}$) as well as a unique more basic phosphoprotein (p72). Both sets of intracellular phosphoproteins may play roles in signalling from the TcR/CD3 complex.

C-MYC IS NECESSARY AND LIMITING FOR TRANSFORMATION OF FIBROBLASTS BY V-ABL AND BCR-ABL. Charles L. Sawyers[1,3], Wendy Callahan[2] and Owen N. Witte[2,3], Departments of [1]Medicine, [2]Microbiology, and [3]Howard Hughes Medical Institute, University of California-Los Angeles, Los Angeles, CA. 90024.

An important role for C-MYC in transformation by ABL tyrosine kinases is suggested by prior work showing upregulation of C-MYC expression in cells transformed by V-ABL and enhanced transformation by V-ABL and BCR-ABL in the setting of C-MYC overexpression. To investigate the role of C-MYC in fibroblast transformation by ABL proteins, we tested a series of cell lines expressing dominant negative mutant human C-MYC proteins for susceptibility to transformation by V-ABL and BCR-ABL using a soft agar transformation assay. Rat-1 fibroblasts expressing MYC mutations in the activation domain or DNA binding domain were resistant to transformation by V-ABL and BCR-ABL, whereas cell lines expressing wild-type C-MYC or a mutation N-terminal to the activation domain enhanced transformation. Suppression of transformation was critically dependent on high level expression of the dominant negative MYC protein. Mutations in the helix-loop-helix or leucine zipper domains, which mediate dimerization with the MYC binding partner MAX, had no effect on ABL transformation, implying that dominant negative MYC mutants exert their effects through binding MAX. These data suggest that transformation of fibroblasts by V-ABL and BCR-ABL occurs through a defined pathway which requires C-MYC as a downstream effector.

IDENTIFICATION OF AN INSULIN RESPONSE ELEMENT WITHIN THE 5' FLANKING REGION OF THE CHICKEN OVALBUMIN GENE. Emmanouil Skoufos, Lora A. Schweers, and Michel M. Sanders. Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455.

The chicken ovalbumin gene is subject to multi-hormonal regulation. Maximal expression requires not only the synergistic effects of corticosterone and estrogen but also the permissive effects of insulin. In an attempt to identify the region that is necessary for the action of insulin, deletion mutants in the 5' flanking sequence of the ovalbumin gene were fused to the chloramphenicol acetyltransferase (CAT) reporter gene. These mutants were tested for inducibility by insulin by transfection into steroid- and insulin-responsive primary oviduct cultures, and expression was measured by CAT assay. The region between -780 and -759 was necessary to convey the effect of insulin and was termed the insulin response element (IRE). Sequence comparisons indicated that sequences similar to the ovalbumin IRE exist in the 5' regions of other genes induced by insulin. Gel retardation studies using the sequence between -776 and -764 indicated that an oviduct nuclear protein binds to this region. The binding of protein to the IRE is enhanced by the presence of insulin in the culture medium of the cells. Treatment of nuclear proteins with phosphatase prior to the binding reaction abolished binding to the IRE. The protein binding to the IRE has previously shown to have functional properties that are similar to NF-κB, whose activity is also regulated by phosphorylation. Thus, insulin modulates the expression of the chicken ovalbumin gene through a pyrimidine-rich response element that is homologous to ones in other insulin-induced genes. Protein phosphorylation is important for interaction of this element with a nuclear protein that may be a member of the NF-κB (*rel* oncogene) superfamily.

PROTEIN CONTENT DISTRIBUTIONS AND SINGLE CELL RATES OF PROTEIN ACCUMULATION IN ASYNCHRONOUS *S. CEREVISIAE* POPULATIONS, Friedrich Srienc, Bruce S. Dien, University of Minnesota--Institute of Advanced Studies in Bioprocess Technology and Department of Chemical Engineering and Materials Science, 240 Gortner Lab, 1479 Gortner Ave., St. Paul, MN 55108

A rigorous description of cell growth within the cell cycle requires knowledge of (i) the overall doubling time of the cell culture, (ii) the pattern of growth rates between cell birth and division, (iii) the probability of a cell to enter the next cell cycle phase, and (iv) the cell partitioning at division. The overall doubling time of a cell population, which is conventionally used to quantify the growth behavior of a cell culture, has the disadvantage of not revealing details of cell proliferation during the cell cycle. We have developed the bromodeoxyuridine labelling and staining methodology to be applicable to *S. cerevisiae* in combination with flow cytometry analysis. It is possible to experimentally determine with this procedure the dynamic characteristics of a growing cell population which includes the growth patterns during the cell cycle, the transition probabilities between individual cell cycle phases, and cell partitioning at division. We have used unperturbed, asynchronous cell populations to investigate how these dynamic characteristics change when the overall growth rate of the cell culture is changed by means of nutritional limitation. The data reveal that daughter cells in G_1 need to obtain a critical protein content before having a non-zero probability of beginning replication of their DNA. Such a threshold protein content was not observed for cells making the transitions between the other cell cycle phases. The data also indicates that there exists a distinct growth pattern during the cell cycle which is characterized by a low rate of single-cell protein accumulation before entering into the DNA replicating cell cycle phase.

A 32Kd GTP-BINDING PROTEIN ASSOCIATED WITH THE CD4:p56^{lck} AND CD8:p56^{lck} COMPLEXES, Janice Telfer and Christopher E. Rudd Division of Tumor Immunology, Dana Farber Cancer Institute, and Department of Pathology, Harvard Medical School, Boston MA 02115

Guanosine triphosphate (GTP) binding proteins include signal transducing heterotrimers (ie. G_s, G_i), smaller GTP-binding proteins that function in protein sorting and oncogenic p21^{ras}. In T-cells, GTP-binding proteins have been indirectly implicated, by cholera toxin inhibition and by G-protein activating reagents, as mediators of TcR:CD3 activation and subsequent phosphatidylinositol turnover. A major issue has been to identify the GTP-binding protein and associated receptor involved in the activation process. Here, we show that the T-cell CD4:p56^{lck} and CD8:p56^{lck} receptor complexes include a 32-33 kD phosphoprotein that can be recognised by an antisera against a consensus GTP-binding region in G-proteins. CD4:p56^{lck} and CD8:p56^{lck} complexes also bound GTP and hydrolysed it to GDP (60 to 70% of bound nucleotide). Nucleotide competition analysis revealed a pattern of inhibition of GTP binding identical to p21^{ras}. CD4:p56^{lck} associated p32 can also be covalently linked to [a-^{32}P] GTP by ultraviolet-photoaffinity labeling. The requirement for the CD4:p56^{lck} complex in optimal functional response of T cells to antigen may be mediated by p56^{lck} and/or p32.

MITOGENICITY OF COLONY STIMULATING FACTOR-1 IN RAT MACROPHAGES REQUIRES PHOSPHATIDYL-CHOLINE HYDROLYSIS AND PROTEIN KINASE C ACTIVATION, V.L. Sylvia, G. Ghosh Choudhury, G. Fernandes and A.Y. Sakaguchi, Depts. of Cellular & Structural Biology and Medicine, U.T. Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284.

The tyrosine kinase colony stimulating factor-1 (CSF1) receptor is necessary for the differentiation and survival of macrophages. Structurally similar to PDGF receptor and the c-*kit* proto-oncogene, CSF1 receptor induces phosphorylation of specific substrates (e.g. phosphatidylinositol 3-kinase) on tyrosine residues. Unlike PDGF receptor, however, activation of the CSF1 receptor does not induce phosphoinositide turnover but instead induces rapid and sustained hydrolysis of membrane phosphatidylcholine (PC) to form diacylglycerol and phosphorylcholine. We tested whether CSF1-stimulated PC hydrolysis in rat macrophages proceeds via tyrosine phosphorylation and whether it is important for subsequent protein kinase C (PKC) activation. Resident peritoneal macrophages isolated from 6 month old male Fischer 344 rats were stimulated with recombinant CSF1 following pretreatment with 100 μM genistein (a tyrosine kinase inhibitor), 20 μM H-7 (a PKC inhibitor), 10 μM U73,122 (a phospholipase C inhibitor) or control RPMI media. ^{3}H-PC hydrolysis, PKC activity and thymidine incorporation assays were performed. Genistein decreased PC hydrolysis by 75%, PKC activity by 92% and DNA synthesis by 66%. H-7 pretreatment had no effect on PC hydrolysis at early time points following CSF1 stimulation, but reduced the sustained accumulation of phosphorylcholine. H-7 inhibited PKC activity by 95% and DNA synthesis by 28%. U73,122 decreased PC hydrolysis by 95%, PKC activity by 88% and DNA synthesis by 89%. Other compounds such as forskolin and pertussis toxin were also tested and their effects will be discussed. The data strongly suggest that the mitogenic effect of CSF1 in rat macrophages relies upon the hydrolysis of phosphatidylcholine. Antibodies prepared against *B. cereus* phosphatidylcholine-specific phospholipase C (PCPLC) will inhibit PC hydrolysis in extracts from CSF1-stimulated rat macrophages. Our laboratory is currently using antiphosphotyrosine and anti-PCPLC immunoaffinity chromatography to purify the mammalian PCPLC involved in CSF1 signal transduction. Supported by the National Institute on Aging, American Cancer Society and the Meadows Foundation.