RECEPTOR-MEDIATED SECOND MESSENGER PATHWAYS

Organizers: Harvey Herschman and Lewis T. Williams

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Receptor-Mediated Second Messenger Pathways

Phospholipase C and PI Signalling

F 001 PHOSPHATIDYLINOSITOL PATHWAYS IN GROWTH AND TRANSFORMATION.
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Two different enzymes which act on polyphosphoinositides have been found to directly associate with and be phosphorylated on tyrosine by the PDGF receptor; PI specific phospholipase C-gamma and Phosphatidylinositol-3 kinase (PI-3 kinase). PI-3 kinase was discovered because of its co-immunoprecipation with the polyoma middle t/pp60$^{c\text{-}src}$ complex (Whitman et al., 1985 Nature 315, 239). The same enzyme was later found to associate with the platelet derived growth factor (PDGF) receptor (Kaplan et al., 1987, CELL 50, 1021). The finding that this enzyme phosphorylates the D-3 position of the myo-inositol moiety of phosphatidylinositol was unexpected and indicated the existence of a new phosphatidylinositol (PI) signal transduction pathway not involving inositol-1,4,5-trisphosphate (Whitman et al., 1988 Nature 332, 644). The purified PI-3 kinase phosphorylates PI, phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate. In all three reactions the phosphate appears to be added at the D-3 position of the inositol ring. Phosphatidylinositol-3-phosphate is present in unstimulated cells at about 3% the level of phosphatidylinositol-4-phosphate and only small changes in its level are observed upon stimulation with PDGF. However, phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-trisphosphate are completely absent in unstimulated cells and appear within a minute of stimulation by PDGF (Auger et al., 1989 CELL 57, 167). Polyoma middle t transformed fibroblasts have elevated levels of PI-3,4-P_2 and PIP_3 even under conditions of serum starvation and high density. Recently we have shown that in addition to the PDGF receptor, two other growth factor receptors in the protein-tyrosine kinase family, the insulin receptor and the colony stimulating factor-1 receptor, physically associate with and activate the PI-3 kinase. The possibility that PI-3 kinase produces second messengers for growth regulation will be discussed.

F 002 EPIDERMAL GROWTH FACTOR DEPENDENT PHOSPHORYLATION OF PHOSPHOLIPASE C-γ, Graham Carpenter, Matthew Wahl, Shunzo Nishibe and William Grizzle, Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232-0146. Epidermal growth factor (EGF) treatment of A-431 cells produces a rapid increase in inositol trisphosphate (IP_3) levels which is independent, initially, of extracellular calcium and subject to modulation by protein kinase C activity (1). Coincident with this metabolic response is the enhanced phosphorylation of a particular phospholipase C isozyme (PLC-γ) (2). Growth factor treatment leads to increased phosphorylation of PLC-γ at multiple tyrosine residues plus a serine residue(s). Specific tyrosine phosphorylation of PLC-γ by the EGF receptor can be demonstrated in vitro, as isoforms PLC-δ and PLC-β do not serve as phosphorylation substrates (3). Interestingly, PLC-γ is unique among the isoforms of PLC in that it contains sequences recognized in other proteins involved in growth regulation as src homology regions. Given this background, more recent data will be presented to deal with various aspects of this potential signaling system for certain growth factors, such as EGF and PDGF.

1. Wahl, M. and Carpenter, G. (1988) J. Biol. Chem. 263: 7581-7590.
2. Wahl et al. (1989) Proc. Natl. Acad. Sci. 86: 1568-1572; Meisenhelder et al. (1989) Cell 57: 1109-1122; Margolis et al. (1989) Cell 57: 1101-1107; Wahl et al. (1989) Molec Cell. Biol. 9: 2934-2943.
3. Nishibe et al. (1989) J. Biol. Chem. 264: 10335-10338.

F 003 REGULATION OF PHOSPHOLIPASE C ISOZYMES BY PROTEIN KINASES, Sue Goo Rhee, Uh-Hyun Kim, Jae Won Kim, and Hee Sook Kim, NIH, NHLBI, Bethesda, MD 20892

Molecular cloning data available up until now suggest that PI-specific phospholipase C (PLC) comprises a large number of super families which can be divided into four subtypes, PLC-α, ß, γ, and δ. The four types of enzymes are quite dissimilar in molecular size: α, 60-68 kDa; ß, 140-150 kDa; γ, 145-148 kDa; and δ, 85-88 kDa. Their primary structures are so different that they are immunologically distinct from each other. The four types of isozymes are expressed differently between tissues, between individual cells, and during development. Thus, different modes of regulation may exist for the isozymes.

It is well established that at least one of the γ type enzymes is directly phosphorylated at multiple sites by growth factor receptor tyrosine kinases. These tyrosine phosphorylations appear to be necessary for the growth factor-dependent stimulation of $InsP_3$ formation.

The formation of $InsP_3$ is significantly inhibited in a variety of tissues and cells pretreated with phorbol ester. In C_6Bu1 and PC-12 cells, both of which contain at least three types of PLC (ß, γ, and δ), only PLC-ß was phosphorylated at serine in response to phorbol ester. This may represent a mechanism by which diacylglycerol, a product of PLC, feedback regulates PLC activity.

There are numerous reports suggesting the existence of cross-talks between cAMP and PI-signaling pathways. Specific phosphorylation of PLC-γ both *in vivo* and *in vitro* by cAMP-dependent kinase (PKA) suggests that the sites of cAMP action includes the phosphorylation of PLC-γ.

Nevertheless, PLC enzymes phosphorylated by either EGF-receptor, PKA, or PKC *in vitro* exhibited no changes in the catalytic activity. This might be due to the fact that the phosphorylations alter PLC activity in a manner dependent on other modulatory protein factors that were not present in the *in vitro* assay system.

Tyrosine Kinase Signalling Mechanisms

F 004 FUNCTIONAL DOMAINS OF THE EPIDERMAL GROWTH FACTOR RECEPTOR, Gordon N. Gill, Department of Medicine, University of California, San Diego, School of Medicine, La Jolla, California 92093

Many growth factors stimulate mitogenesis by binding to members of a family of cell surface receptors that possess protein tyrosine kinase activity. The mechanism of signal transduction and specifically the role of ligand-induced receptor internalization in signal transduction have remained undefined. We have located the distal boundary of the tyrosine kinase domain of the EGF receptor and have shown that this corresponds to an intron-exon boundary in the gene. The C' terminal domain distal to this boundary contains four identified sites of tyrosine self-phosphorylation. The C' terminus is proposed to be a regulatory domain with self-phosphorylation at these sites removing an alternate substrate/inhibitory constraint. C' terminal truncated EGF receptors lacking the region of self-phosphorylation demonstrate EGF-dependent tyrosine phosphorylation of cell substrates, supporting the hypothesis that self-phosphorylation is limited to the regulatory C' terminus and that non-self-phosphorylated EGF receptors are active in vivo. The distal part of the C' terminus is the primary inhibitory domain. A distinct sequence proximal to this is required for EGF-dependent receptor internalization leading to down-regulation and degradation. Within this receptor domain an 18 amino acid highly negatively charged region of predicted helical structure bounded by turns is required for both endocytosis via a high affinity saturable pathway and for ligand-stimulated increases in cytosolic calcium. Because of its role in increasing cell calcium and in receptor internalization, this domain is referred to as the CaIn domain. Identification of this domain allowed generation of kinase active internalization defective receptors. When transfected into null recipient B82 and NR6 cells, these effectively signaled gene transcription and growth. The dose-response curves for growth were shifted to the left approximately one order of magnitude as would be predicted for a nonattenuating receptor. The receptors lacking the CaIn domain demonstrated ligand-dependent formation of transformed foci in monolayer culture and of colonies in soft agar. Studies with these C' terminal truncated receptors support the hypothesis that the mitogenic responses to EGF are mediated via activation of the intrinsic protein tyrosine kinase activity of the membrane bound receptor with ligand-induced internalization serving to attenuate signaling. In the absence of attenuation, enhanced growth responses and transformation result.

F 005 PROTEIN-TYROSINE KINASES AND THEIR TARGETS, Tony Hunter, Kathy Gould, Rick Lindberg, Dave Middlemas, Jill Meisenhelder, and Peter van der Geer, Molecular Biology and Virology Laboratory, The Salk Institute, P.O. Box 85800, San Diego, CA 92138

Protein phosphorylation is a major mechanism whereby signals are transduced from external stimuli into cellular responses. Many growth factor receptors are ligand-activated protein-tyrosine kinases (PTK), while other PTKs, such as $pp60^{c\text{-}src}$, located on the inside of the plasma membrane may also be involved in signal transduction. We have identified novel PTKs by screening cDNA libraries with oligonucleotide probes to consensus sequences. In this way we have isolated are 2 novel receptor-like PTKs. One of these, *eck*, was isolated from a HeLa cell library. *eck* RNA is predominantly expressed in tissues containing proliferating epithelial cells (skin, lung and intestine), and in epithelially-derived cell lines. The predicted *eck* protein, has 976 residues, and is closely related to the *eph* and *elk* receptor-like PTKs. Antibodies raised against a TrpE-*eck* fusion protein immunoprecipitate a 125 kDa protein from epithelial cell lines. This protein is phosphorylated on tyrosine in an immune complex kinase assay, indicating that the *eck* protein is a PTK. Immunostaining methods are being used to localize the *eck* protein in tissue sections. In collaboration with Dennis Slamon (UCLA) we have found that the *eck* gene is amplified in about 20% of human breast carcinomas. A second putative PTK, *trk*-B, was isolated from a rat cerebellar library. The predicted *trk*-B protein has 810 amino acids, and it is closely related to but distinct from the *trk* receptor-like PTK. *trk*-B is exclusively expressed in brain, as a series of RNAs ranging from 13 to 0. 8 kb. The smaller RNAs are clearly too short to encode the intact protein. Analysis of additional *trk*-B cDNAs indicates that there are mRNAs which encode a protein truncated just downstream of the transmembrane domain, with a short novel C terminus. We are currently investigating the distribution of the two types of mRNA in the brain, and trying to identify their protein products, and determine whether the full length protein has PTK activity. We have also begun to analyse phosphorylation sites in the CSF-1 receptor, a well-characterized PTK receptor. We have identified Tyr 706 in the kinase insert, as a major site of CSF-1-induced autophosphorylation. There is no evidence that Tyr 969 near the C terminus is phosphorylated. We are assessing the effects of mutation of Tyr 706 on CSF-1 receptor function.

To learn more about signal transduction mechanisms we are attempting to identify substrates for PTKs. Several substrates associated with the submembraneous cytoskeleton have been identified. Ezrin (p81), a substrate for both the EGF receptor and $pp60^{v\text{-}src}$ PTKs, resides in the core of surface microvilli. With Tony Bretscher (Cornell), we have isolated a full length human ezrin cDNA clone. The predicted sequence of ezrin p81 shows that it is likely to have globular regions at its N and C terminus connected by an α-helical region. The N-terminal domain has 37% identity to the N-terminal domain of the red cell cytoskeletal protein, band 4.1, which binds to glycophorin via this region. We are carrying out site-directed mutagenesis to determine whether this region of ezrin has a similar function. In collaboration with Sue Goo Rhee (NIH) we have recently identified phospholipase C-γ (PLC-γ) as a substrate for the PDGF and EGF receptors in vivo and in vitro. PLC-γ is rapidly and extensively phosphorylated on Tyr and Ser when quiescent NIH 3T3 cells are treated with PDGF, and this may account for the PDGF-induced PI turnover. A fraction of PLC-γ is found associated with the activated PDGF receptor. We are currently analysing a series of additional proteins that are found in a complex with PLC-γ.

F 006 TRANSFORMING POTENTIAL OF GROWTH FACTOR RECEPTORS, Axel Ullrich[1], Robert Hudziak[2], Sharon Massoglia[2], James Lee[2], Dennis Slamon[3], and Joseph Schlessinger[4], [1]Max-Planck-Institut für Biochemie, 8033 Martinsried, West Germany; [2]Genentech, Inc., South San Francisco, CA 94080; [3]Division of Hematology-Oncology, UCLA School of Medicine, Los Angeles, CA 90024; [4]Rorer Biotechnology, King of Prussia, PA 19406

Using cloned growth factor receptor cDNAs and structural information of both normal receptors and their oncogenic derivatives, we have investigated alternative scenarios that result in receptor-mediated cellular transformation. We find that certain structural alterations, including truncations, deletions and point mutations, in the EGF receptor can contribute to the induction of a transformed phenotype on transfected NIH 3T3 cells. Furthermore, increased expression levels in conjunction with activation by the ligand results in efficient transformation by epidermal growth factor receptor and HER2/*neu*. Growth factor receptor gene amplification and overexpression appears to be a frequent characteristic of primary mammary carcinomas and correlates statistically with patient survival and tumor recurrence.

Various approaches designed to analyze basic mechanisms underlying normal growth factor receptor-mediated signalling and their subversion in the transformed cell and in malignant cancer will be discussed.

Signalling in the Immune System

F 007 CD4 AND CD8 SIGNAL TRANSDUCTION IN HUMAN T LYMPHOCYTES

Peter A. Thompson[1,3], Ivan D. Horak[1,2], Anne L. Burkhardt[1,2], Eva M. Horak[1], and Joseph B. Bolen[1], Laboratory of Tumor Virus Biology[1], Medicine Branch[2], and NCI-Navy Medical Oncology Branch[3], National Cancer Institute, Bethesda, Maryland 20892.

Mature T lymphocytes can be divided into two mutually exclusive subsets based upon the expression of the CD4 and CD8 surface glycoproteins. While CD4+ T-cells generally possess a helper phenotype and CD8+ T-cells generally possess a cytotoxic phenotype, CD4 and CD8 expression physiologically correlates with the capacity of a given T-cell to interact with non-polymorphic determinants of class (CD8) and class II (CD4) molecules on the surface of antigen presenting cells - an event thought to normally be critical for T-cell activation. Several lines of evidence suggest that CD4 and CD8 are capable of generating signals in T-cells that are important for T-cell receptor dependent activation events. The most compelling support for this view is the observation that both CD4 and CD8 are associated with the lymphocyte-specific tyrosine protein kinase, $p56^{lck}$. These non-covalent complexes have been demonstrated to be capable of signal generation as evidenced by increased $p56^{lck}$ protein kinase activity and rapid tyrosine phosphorylation of several T-cell proteins following CD4 engagement on the cell surface. One of the putative target proteins for $p56^{lck}$ has been shown to be the ζ subunit of the T-cell receptor thereby providing an example of how CD4 might transmit a signal to other membrane proteins through the function of its associated kinase molecule. However, the variety of responses by T-cells to CD4 and CD8 surface engagement thought to be independent of T-cell receptor mediation indicate that defining additional substrates for the CD4-$p56^{lck}$ and CD8-$p56^{lck}$ complexes is important for our understanding of the full range of CD4- and CD8-dependent signals. The identification and characterization of several of these putative substrates will be discussed as will other factors that appear to independently regulate the activity of $p56^{lck}$ in T-cells.

F 008 T-CELL ACTIVATION: MULTIPLE KINASES CONTROLLED BY A COMPLEX SIGNALLING APPARATUS, Richard D. Klausner, Stuart Frank, Jonathan Ashwell, Jeffrey Siegel and Lawrence E. Samelson, Cell Biology and Metabolism, NICHD, National Institutes of Health, Bethesda, Maryland.

The physiologic activation of T lymphocytes is an extremely complicated process involving the interaction of an antigen presenting cell with surface molecules present on the T cell. Many of these interactions are mediated via the T cell antigen receptor complex (TCR). This receptor is a complex of at least seven transmembrane chains composed of three distinct genetic groups of subunits. One group is responsible for ligand recognition, the other two groups, CD3 and zeta/eta are presumably involved in activation of the receptor and coupling to signal transduction pathways. We have been recently performing reconstitution and mutagenesis studies of the receptor complex in order to model information flow through the complex. This has allowed to us to propose a unique role for the zeta homodimer in tranducing the signal produced by receptor occupancy to the CD3 coupling complex. In addition the presence of two distinct subsets of receptors determined by the presence or absence of a zeta/eta heterodimer has been correlated with coupling to at least two distinct receptor pathways. Receptors containing eta are required for coupling to phosphatidylinositide hydrolysis while eta is not required for the activation of a receptor associated tyrosine kinase. The phosphatidylinositide pathway activates protein kinase C and this is responsible for the phosphorylation and activation of the C-raf proto-oncogene. In addition a non-PKC, serine kinase pathway that does not involve the TCR results in activation of C-raf as a consequence of presenting cell/ T cell interaction. We have recently identified the c-fyn proto-oncogene as the tyrosine kinase most likely to be directly activated by the receptor. This is demonstrated by a physical association between the receptor and c-fyn and the ability of receptor/c-fyn complexes to tyrosine phosphorylate the receptor in vitro. Multiple cellular substrates are phosphorylated on tyrosine residues in response to T cell activation and differences in both the kinetics and pharmacology of phosphorylation of substrates suggest a complex series of tyrosine kinase activation pathways. Finally T cell activation results in unusual intracellular redistribution of the CD45 tyrosine phosphatase and the implications of this for signal transduction will be discussed.

F 009 THE ROLE OF CD45 (T200, L-CA) IN LYMPHOCYTE FUNCTION, Ian S.Trowbridge, Hanne L. Ostergaard, Deborah A. Shackelford, Tamara R. Hurley, Pauline Johnson, Robert Hyman and Bartholomew M. Sefton, Department of Cancer Biology and Molecular Biology and Virology Laboratory, The Salk Institute, La Jolla, CA 92037

CD45 is a family of major leukocyte-specific cell surface glycoproteins expressed exclusively on hematopoietic cells. Isoforms of CD45 are generated by the alternative splicing of three exons, each encoding ≈ 50 amino acids that are inserted near the amino terminus of the molecule. Different isoforms of CD45 are selectively expressed on specific subpopulations of hematopoietic cells, and changes in the pattern of expression of CD45 isoforms in T cells also occur upon antigenic stimulation. CD45 is also distinguished by a large, highly conserved, cytoplasmic domain of 705 amino acids that can be subdivided into two related subdomains of ≈ 300 amino acids. Recently, each of these subdomains was shown to have 30-40% identity with a soluble human placental protein-tyrosine phosphatase (PTPase) and CD45 isolated from human spleen was found to have intrinsic PTPase activity[1,2]. We have analyzed the PTPase activity of individual isoforms of murine CD45 and truncated forms of the molecule[3]. Each of the known isoforms of murine CD45 has an equivalent basal level of PTPase activity and the cytoplasmic domain of CD45 produced as a secreted protein in a baculovirus system has intrinsic enzymatic activity. Further, the availability of three independent sets of parental CD45+, mutant CD45-, and revertant CD45+ lymphoma cells has allowed us to search for *in vivo* substrates of the CD45 PTPase activity by comparing the phosphotyrosine-containing proteins in CD45+ and CD45- cells. The results of this analysis indicate that loss of CD45 in the mutant lymphoma cells correlates with increased phosphorylation of the *src*-related leukocyte-specific tyrosine protein kinase p56^{lck} at Tyr 505, a putative negative regulatory site. Previous studies using monoclonal antibodies have implicated CD45 in a variety of lymphocyte functions including proliferative responses and signal transduction. Our data suggest that one possible mechanism by which CD45 might play a role in lymphocyte growth regulation is by directly, or indirectly, modifying the kinase activity of p56^{lck}. The level of expression of CD45 does not change dramatically during hematopoietic development implying that its PTPase activity is subject to regulation. The structure of CD45 is analogous in many respects to that of the growth factor protein tyrosine kinase receptors suggesting that it plays a similar role in signal transduction through regulation of its PTPase activity by an external ligand. It is probable that CD45 is member of a family of related receptor PTPases some of which may be found widely distributed in a variety of tissues and organisms.

1. Charbonneau, H., Tonks, N. K., Walsh, K. A., and Fischer, E. H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 7182-7186.
2. Tonks, N. K., Charbonneau, H., Diltz, C. D., Fischer, E. H. and Walsh, K. A. (1988) *Biochemistry* **27**, 8696-8701.
3. Ostergaard, H.L., Shackelford, D. A., Hurley, T. R., Johnson, P. Hyman, R., Sefton, B. M. , and Trowbridge I. S. (1989) *Proc Natl. Acad. Sci. USA* **86**, in press.

Serine Threonine Protein Kinases

F 010 THE REGULATION OF pp90rsk A HIGHLY CONSERVED MITOGEN-RESPONSIVE SERINE-SPECIFIC PROTEIN KINASE, Raymond L. Erikson, David Alcorta, Laurel J. Sweet, Timothy J. Martins and Terry Vik, Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138. Reversible protein phosphorylation is believed to play a major role in the response of cells to mitogens. Although in many cases the initial event involves the activation of tyrosine-specific protein kinases this signal rapidly results in the activation of serine/threonine-specific protein kinases. Indeed in vivo tyrosine phosphorylation of proteins is rarely seen in the absence of additional modification by serine/threonine phosphorylation. We are interested in the molecular connection between the two major classes of protein kinases and have obtained cDNA clones for a major serine-specific enzyme originally identified as a ribosomal protein S6 kinase (rsk) (1,2,3). The product of this gene, pp90rsk, and a Mr=65,000-70,000 enzyme appear to be major contributors to S6 phosphorylation in cultured cells. Recombinant baculoviruses have been used to express rsk products in insect cells with or without gene products that may activate the enzyme. We find that v-src has the capacity to achieve at least a 5,000 fold activation of pp90rsk. Under these conditions, pp90rsk is phosphorylated on tyrosine but we have no evidence this has physiological relevance. In this regard a kinase activity has been identified in mitogen-stimulated chicken cells that phosphorylates pp90rsk on threonine residues in a manner similar to MAP kinase (4). The mechanisms of rsk activation and its potential to phosphorylate other substrates will be discussed.

1. Erikson, E. and Maller, J.L., Proc. Natl. Acad. Sci. USA **82**:742-746, 1985.
2. Jones S.W., Erikson, E., Blenis, J., Maller, J.L. and Erikson, R.L., Proc. Natl. Acad. Sci. USA **85**:3377-3381, 1988.
3. Alcorta , D. Crews, C.M., Sweet, L.J., Bankston, L., Jones, S.W. and Erikson, R.L., Mol. Cell. Biol. **9**:3850-3859, 1989.
4. Sturgill, T.W., Ray, L.B., Erikson, E., and Maller, J.L., Nature (Lond.) **334**:715-718, 1988.

Signalling in Single Cell Organisms

F 011 POSTTRANSLATIONAL MODIFICATIONS AT THE C-TERMINUS OF SIGNALLING PROTEINS
Steven Clarke, Department of Chemistry and Biochemistry and the Molecular Biology Institute, UCLA, Los Angeles, CA 90024

In collaboration with Drs. Robert Deschenes (Univ. Iowa) and Jeffry Stock (Princeton Univ.), we have studied the structure and function of covalent modifications of ras oncogene products that occur at the carboxyl-terminus. We originally hypothesized that the lipidation, methyl esterification, and proteolytic cleavage reactions involved in the maturation of fungal peptidyl sex factors would also occur in proteins that had similar Cys-Xaa-Xaa-Xaa C-terminal sequences (1). We demonstrated that the mammalian Ha-ras and the yeast RAS2 products are methylated in reactions consistent with the esterification of C-terminal cysteine residues (1,2). Work in other laboratories has supported this hypothesis (3,4) and has shown that the lipidation reactions may involve both fatty acyl and isoprenoid groups (4). We are presently determining the complete covalent structure of the C-terminus of the yeast RAS2 protein as a step in the elucidation of the physiological role of these modifications in the signalling function of the ras proteins.

The cGMP phosphodiesterase of retinal rods also contains a initial Cys-Xaa-Xaa-Xaa C-terminal sequence. In collaboration with Dr. Bernard Fung (UCLA), we have now established that this enzyme is also methyl esterified at a C-terminal cysteine residue and that this region is involved in its binding to the membrane (5). We proposed that the methylation and the possible lipidation of this protein may play a crucial role in positioning it in the rod outer segment disks for interacting with transducin in the phototransduction process. Finally, evidence has been obtained for a similar type of methylation reaction in a small molecular weight G-protein from brain (6) and in a potentially similar protein from retinal rods (7).

1. Clarke, S., Vogel, J.P. Deschenes, R.J., and Stock, J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 4643-4647.
2. Deschenes, R.J., Stimmel, J.B., Clarke, S., Stock, J., and Broach, J.R. (1989) *J. Biol. Chem.* **264**, 11865-11873.
3. Anderegg, R.J., Betz, R., Carr, S.A., Crabb, J.W., and Duntze, W. (1988) *J. Biol. Chem.* **263**, 18236-18240.
4. Hancock, J.F., Magee, A.I., Childs, J.E., and Marshall, C.J. (1989) *Cell* **57**, 1167-1177.
5. Ong, O. C., Ota, I. M., Clarke, S., and Fung, B. K.-K. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, in press.
6. Yamane, H. K. and Fung, B. K.-K. (1989) *J. Biol. Chem.* **264**, in press.
7. Ota, I. M. and Clarke, S. (1989) *J. Biol. Chem.* **264**, 12879-12884.

F 012 TRANS-ACTING FACTORS REQUIRED FOR PEPTIDE HORMONE-INDUCED GENE EXPRESSION IN YEAST, Frederick S. Gimble, Jennifer L. Davis, Miriam S. Hasson, Elisabeth T. Barfod and Jeremy Thorner, Division of Biochemistry and Molecular Biology, University of California, Berkeley CA 94720. Mating between haploid cells of the yeast *Saccharomyces cerevisiae* requires induction by peptide mating pheromones of the expression of a number of genes whose products are essential for the mating process. We have shown previously that a tandem repeat of an 8-bp sequence is both necessary and sufficient to serve as a pheromone-dependent upstream activation sequence. We have termed this 8-bp site the "pheromone response element" (or PRE). We have identified an activity in yeast extracts that binds to the PRE in a sequence-specific manner and have purified this factor, which we have called the PRE-binding factor (or PBF), to near-homogeneity by ammonium sulfate fractionation and subsequent chromatography on heparin-agarose, Cibacron blue-agarose, and an oligonucleotide affinity resin. Purified PBF is a 50,000 MW protein whose specific binding to PREs can be detected by gel mobility shift, by covalent cross-linking to a 5 BrdU-containing PRE, and by probing of protein blots with labelled PRE sequences. PBF binds directly to PRE sites, as demonstrated by DNAse I protection assays. PBF binding activity is found at essentially equivalent levels in both haploid cell types and in diploids. At least one other transcription factor required for expression of cell type-specific genes, the *MCM1* gene product, is also found in all three cell types. PBF level does not appear to change in response to pheromone stimulation; covalent modification of PBF has not yet been examined. PBF can be purified from mutant cells that lack either functional *STE12* or *STE5* gene products, which are candidate DNA-binding proteins and required for basal expression and pheromone induction of cell type-specific genes. One of these gene products, *STE12*, has been shown to encode a protein that is capable of binding to PRE sequences (Dolan et al., 1989; Errede & Ammerer, 1989). PBF may act as a repressor to prevent occupancy of PRE sites by *STE12* product; alternatively, hetero-oligomers of PBF and *STE12* may exhibit a higher affinity for PRE sites and/or greater transcriptional activation capacity. Overproduction of either *STE5* product (or *STE12* product; S. Fields, personal communication) leads to elevated expression of pheromone-inducible genes, even in the absence of hormone, suggesting that these factors are primary mediators of the distal end of the pheromone response pathway. Supported by Damon Runyon Postdoctoral Fellowship DRG909 to F.S.G., by American Cancer Society Postdoctoral Fellowship PF3308 to J.L.D., by NIH Predoctoral Traineeship GM08295 to M.S.H., by NIH Postdoctoral Traineeship GM09041 to E.T.B., and by NIH Research Grant GM21841 to J.T.

Mitogenesis

F 013 G-PROTEIN COUPLED RECEPTORS AND GROWTH CONTROL - A REEVALUATION OF THE ROLE OF POLYPHOSPHOINOSITIDE BREAKDOWN. Jacques POUYSSEGUR, Catherine KAHAN and Klaus SEUWEN, *Centre de Biochimie du CNRS, Faculté des Sciences, Parc Valrose 06034 Nice - France.*

Receptor-tyrosine kinases, G-protein coupled receptors and ligand-activated ionic channels represent three major classes of transmembrane signalling molecules. The first two are involved in signalling cell growth. Although there is no doubt that receptor and non-receptor tyrosine kinases play a key role in initiating cell cycle entry and sustaining cell proliferation, the nature of the G-protein controlled effector-systems that stimulate DNA synthesis in response to thrombin, serotonin, bombesin, angiotensin, histamine, etc., in various cell types, is not fully understood. These growth promoting agents share in common the capacity to activate polyphosphoinositide specific phospholipase C (PIP_2-PLC), an effector system generally accepted as determinant in growth stimulation. However, because a given hormone often "co-activates" several effector systems, probably *via* distinct receptor subtypes, we consider that the real contribution of activated PIP_2-PLC in growth control has not yet been resolved with certainty. To examine this question, we decided to reconstitute, step by step, signalling pathways which trigger DNA replication by transfection of single G-protein coupled receptor genes: α_2-adrenergic-R coupled negatively to adenylyl cyclase (AC) *via* Gi, and muscarinic HM1-R and serotoninergic 5-HT1c-R both coupled to PIP_2-PLC *via* Gp. These receptors were stably expressed in the Chinese hamster lung cell line, CCL39, that does not respond to adrenergic or muscarinic agonists and that can be growth-arrested in G0 efficiently.

First, we show that the Gi-activated pathway (measured by inhibition of AC) is essential for the mitogenic action of serotonin, thrombin or epinephrine in α_2-R-expressing cells. However, alone, this pathway has no effect on cell cycle entry.

Second, we show that in HM1-R- and 5-HT1c-R-expressing cells, carbachol or serotonin, like thrombin, can induce a strong PIP_2-PLC activation and subsequent Ca^{++} transient, pHi rise and early immediate gene transcription (fos, c-jun). However, in contrast to thrombin, carbachol- or serotonin-stimulated cells are not able to enter the cell cycle. Furthermore, persistent PIP_2-PLC activation does not prevent cycling cells from returning to the growth-arrested state G0 upon serum deprivation.

Therefore we conclude that alone, or in combination, the Gi- and Gp-activated pathways cannot drive cells into the cell cycle. Activation of a tyrosine kinase (EGF-R, FGF-R) is required to reveal the mitogenic potential of these effector systems. Consequently, besides Gi- (↓ AC) and Gp-(↑ PLC) activated pathways, thrombin must activate an additional effector system which permits G0-arrested cells to progress into the S-phase. We favor the idea that this effector is a tyrosine kinase.

F 014 SIGNAL TRANSDUCTION BY NORMAL AND ONCOGENIC ALLELES (c-fms) ENCODING THE HUMAN COLONY-STIMULATING FACTOR 1 RECEPTOR (CSF-1R). Charles J. Sherr,[1,2] Gary Borzillo,[2] Jun-ya Kato,[1,2] James R. Downing,[2,3] and Martine F. Roussel,[2] Howard Hughes Medical Institute[1] and Depts. of Tumor Cell Biology[2] and Pathology,[3] St. Jude Children's Research Hospital, Memphis, Tennessee 38105

In adult animals, the receptor for CSF-1 is restricted in its expression to monocytes, macrophages, and their committed bone marrow progenitors. Transduction of the human c-fms proto-oncogene into PDGF-dependent murine fibroblasts, interleukin-3 dependent myeloid cells, and stromally-dependent immature B cells in long term culture enables them to proliferate in response to human recombinant CSF-1. Therefore, the lineage specificity of the normal CSF-1 response is primarily regulated through receptor synthesis and not by other interacting components of the mitogenic machinery which are more ubiquitously expressed. A consequence is that the v-fms oncoprotein, a mutated form of CSF-1R that exhibits ligand-independent tyrosine kinase activity, can transform many different cell types, ultimately inducing the formation of factor-independent, tumorigenic variants by a nonautocrine mechanism. A serine to leucine substitution at codon 301 in the extracellular domain of human CSF-1R is sufficient to activate its ligand-independent transforming activity. Glutamic acid, threonine, or proline substitutions at position 301 each induced ligand-independent transformation, whereas methionine, cysteine, phenylalanine, or lysine replacements did not. Studies with an independent sarcoma virus isolate suggest that mutations at alternative sites can substitute for codon 301 mutations in inducing oncogenic transformation. A kinase-defective receptor containing a methionine for lysine substitution at its ATP binding site [CSF-1R(met616)] was nonmitogenic and did not undergo downmodulation in response to CSF-1. In contrast, CSF-1R(met616) was phosphorylated on tyrosine in trans and rapidly downmodulated when coexpressed in vivo with enzymatically-competent CSF-1R, implying that CSF-1 induces receptor aggregation, cross-phosphorylation of CSF-1R monomers, and internalization of stably associated complexes. Activation of the CSF-1R kinase leads to the phosphorylation of a series of cellular substrates on tyrosine, at least some of which are important for mitogenesis. Unlike the PDGF and EGF receptors, CSF-1R does not phosphorylate phospholipase C-γ_1, or mobilize soluble inositides and calcium. In contrast, agents that induce protein kinase C activate a protease that cleaves CSF-1R near its transmembrane segment, releasing the intact ligand-binding domain from the cell and generating a fragment representing the kinase domain which is rapidly degraded. We suggest that transmodulation of CSF-1R during macrophage activation precludes their mitogenic response to CSF-1 and limits their survival at sites of inflammation.

Signalling by Cell-Cell Contact

F 015 REGULATION OF EARLY XENOPUS DEVELOPMENT BY GROWTH FACTORS: SENSE AND ANTI-SENSE

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Pre-ectodermal blastomeres from a Xenopus embryo can be converted to mesoderm by the addition of basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β). Among the members of the TGF-β family, TGF-β2 is able to induce mesoderm whereas TGF-β1 can not induce mesoderm but can function synergistically with bFGF to enhance the formation of mesodermal cell types. bFGF and its receptor are present in the early embryo, strongly suggesting that bFGF is a natural mesodermal inducer. However the presence of the bFGF protein in the embryo prior to cellularization, and the lack of a secretory signal sequence in the amino acid sequence of bFGF, raise many questions as to its mechanism of action during embryogenesis.

Three transcripts are produced from the bFGF gene in the oocyte. The predominant transcript is transcribed in the opposite direction to that encoding bFGF. This "anti-sense" RNA causes the covalent modification of the transcript encoding bFGF during the maturation of the oocyte converting approximately half of the adenosine residues to inosine in the region where the two transcripts overlap. The function of this modification is still not understood, but may be related to the degradation of the bFGF transcript during this stage of development. In addition, the anti-sense transcript encodes a putative 25 kd protein that appears to be highly conserved in mammals. This protein has no homolog in the sequence database.

Signalling Mechanisms with G Proteins, G Protein-Coupled Receptors and Phospholipase C and PI Mechanisms

F 100 SELECTIVE INHIBITION OF BOTH THE PDGF RECEPTOR TYROSINE KINASE AND PDGF-DEPENDENT PHOSPHOLIPASE C ACTIVATION BY STAUROSPORINE IN SWISS 3T3 CELLS.

J. Paul Secrist, Inder Sehgal, Richard Olsen, Garth Powis, and Robert T. Abraham. Departments of Immunology and Pharmacology, Mayo Clinic/Foundation, Rochester, MN 55905.

The binding of PDGF to specific receptors (PDGF-R) expressed on quiescent fibroblasts initiates the generation of transmembrane signals that culminate in cellular proliferation. Proximal events linked to mitogenic signal transmission from ligand-bound PDGF-R are stimulation of the receptor tyrosine kinase activity and phosphoinositide hydrolysis due to activation of phospholipase C (PLC). Recent data indicate that PLC-γ is a substrate for the PDGF-R tyrosine kinase; however, the relationship between tyrosine phosphorylation on PLC-γ and PLC activation by PDGF remains unclear. In this study, we demonstrate that the protein kinase inhibitor, staurosporine, is a potent inhibitor of the PDGF-R tyrosine kinase activity in Swiss mouse 3T3 fibroblasts. Staurosporine (0.1-1 μM) pretreatment inhibits PDGF-dependent tyrosine phosphorylation of both the PDGF-R and PLC-γ. Furthermore, staurosporine concomitantly blocks PDGF-dependent PLC activation and the subsequent increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). In contrast, the PLC activation and $[Ca^{2+}]_i$ increase induced by bradykinin, which do not involve tyrosine kinase activation, are staurosporine-insensitive. Thus, staurosporine is a selective inhibitor of both the phosphorylation of PLC on tyrosine residues and the stimulation of phosphoinositide hydrolysis induced by ligand-bound PDGF-R . These data strongly suggest a causal relationship between PLC-γ phosphorylation and PLC activation in PDGF-stimulated fibroblasts. Supported by CA42286 (GP) and DCB-8616001 (RTA).

F 101 $Ins(1,3,4,5)P_4$ INDUCES THE REUPTAKE OF Ca^{2+} RELEASED BY $Ins(2,4,5)P_3$ IN THE SEA URCHIN TRYPTNEUSTES GRATILLA, Alton L. Boynton and Timothy D. Hill, Cancer Research Center of Hawaii, University of Hawaii, Honolulu, HI 96813.

$Ins(1,3,4,5)P_4$ is derived from phosphorylation of the second messenger $Ins(1,4,5)P_3$ by a Ca^{2+}/calmodulin dependent kinase. $Ins(1,4,5)P_3$ releases Ca^{2+} from intracellular stores in both mammalian and most non-mammalian cells. However, the physiological role of $Ins(1,3,4,5)P_4$ is still not fully understood although we have recently demonstrated that it induces the sequestration of Ca^{2+} into an $Ins(1,4,5)P_3$ sensitive pool using electropermeable cells. Our results demonstrate that in sea urchin eggs microinjected with fura 2 that $Ins(1,4,5)P_3$ induces the release and sequestration of Ca^{2+} while $Ins(2,4,5)P_3$ induces only the release of Ca^{2+} presumably due to its inability to be metabolized. However, the microinjection of both $Ins(2,4,5)P_3$ and $Ins(1,3,4,5)P_4$ induces the release and sequestration of Ca^{2+} in a time frame similar to the response elicited by $Ins(1,4,5)P_3$. Thus, $Ins(1,3,4,5)P_4$ stimulates the removal of intracellular Ca^{2+} into internal Ca^{2+} pools and thus a high intracellular Ca^{2+} concentration is returned to resting levels. The Ca^{2+} removed from the cytosolic compartment might be used to refill storage pools sensitive to $Ins(1,4,5)P_3$ as shown in mammalian cells.

F 102 ELEVATED LEVELS OF INSULIN-SENSITIVE GLYCOSYLPHOSPHATIDYL-INOSITOL IN RAS TRANSFORMED RAT-1 CELLS,

B.M.T. Burgering[1], S. Alemany[2], J.M. Mato[2], J.L. Bos, [1]Department for Molecular Carcinogenesis, Sylvius Laboratory, Leiden, The Netherlands, [2] Departemento de Metabolismo, Nutrición and Hormonas, Fundación Jimenez Diaz, Madrid, Spain.

Cells transformed by oncogenes often display alterations in cellular concentration of phospholipids, thought to be involved in signal transduction mechanisms. These changes may indicate the involvement of these oncogenes in particular growth factor-induced signal transducing pathways. We have already shown a possible involvement of normal H-ras in the insulin signalling pathway and to extend this observation we investigated the metabolism of the insulin-sensitive glycosyl phosphatidylinositol (PI-Gly) in ras-transformed rat-1 cells. Analysis of differentially transformed rat-1 cells revealed that (1) the basal level of PI-gly is elevated in ras-transformed cells, and (2) the elevation correlates with the degree of transformation. These and other results presented provide an example of the biochemical level at which ras-function and insulin signal transduction might be connected.

F 103 EXAMINATION OF BRADYKININ-INDUCED $[Ca^{2+}]_i$ CHANGES IN INDIVIDUAL HUMAN FIBROBLASTS BY IMAGE ANALYSIS, Kenneth L. Byron and Mitchel L. Villereal, Dept. of Pharmacological and Physiological Sciences, University of Chicago, Chicago, IL 60637. Image analysis of fura-2-loaded human foreskin fibroblasts was utilized to examine temporal changes in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in individual cells stimulated with lys-bradykinin (BK). Two components of BK-induced $[Ca^{2+}]_i$ elevation, release of intracellular Ca^{2+} stores and entry of extracellular Ca^{2+}, can be distinguished: the Ca^{2+} entry component is blocked by removal of external Ca^{2+} or by the addition of Ni^{2+}, while release of intracellular Ca^{2+} occurs in the presence of Ni^{2+} or absence of extracellular Ca^{2+}. BK-sensitive Ca^{2+} stores are emptied by exposure of the cells to BK in Ca^{2+}-free medium: a second dose of BK in Ca^{2+}-free medium produces no elevation of $[Ca^{2+}]_i$. However, refilling of the Ca^{2+} stores can be accomplished by exposure of these cells to a pulse of extracellular Ca^{2+}. This produces an elevation of $[Ca^{2+}]_i$ which is sustained for the duration of the pulse. Following this treatment, a BK-induced Ca^{2+}-release can again be demonstrated in Ca^{2+}-free medium. Although extracellular Ca^{2+} is required for refilling of the BK-sensitive Ca^{2+} stores, an elevation of $[Ca^{2+}]_i$ is apparently not required. Ni^{2+} completely blocks the elevation of $[Ca^{2+}]_i$ during the refilling period without inhibiting refilling of the BK-sensitive pool: when cells are refilled with Ca^{2+} in the presence of Ni^{2+}, subsequent exposure to BK in Ca^{2+}-free medium induces an elevation of $[Ca^{2+}]_i$. Supported by NIH grant GM-28359 and Training Grant 5T32 GM-07151.

F 104 **PURIFICATION OF PHOSPHOINOSITIDE-3 KINASE, Christopher L. Carpenter, Kurt R. Auger and Lewis C. Cantley, Department of Physiology, Tufts University School of Medicine, Boston, MA 02111. Phosphoinositide-3 kinase (PI-3 kinase) is a crucial enzyme in signal transduction in cells transformed by v-src or polyoma middle T antigen and in the cellular response to PDGF or insulin stimulation. Previous work has implicated an 85kd phosphoprotein as PI-3 kinase. Using a six step procedure we have purified PI-3 kinase from rat liver. The preparation is purified several thousand fold and phosphorylates phosphatidylinositol, phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate. We have renatured these activities from an SDS-PAGE gel.**

F 105 PERTUSSIS TOXIN EXPRESSION IN T CELLS OF TRANSGENIC MICE Karen E. Chaffin, Chan R. Beals, Thomas M. Wilkie*, Melvin I. Simon* and Roger M. Perlmutter, Howard Hughes Medical Institute, University of Washington, Seattle, WA 98195; *California Institute of Technology, Pasadena, CA 981125. Because Ca^{2+} mobilization and increases in phospholipase C (PLC) activity are associated with stimulation of the T lymphocyte antigen receptor/CD3 (TCR/CD3), it has frequently been suggested that all or part of the signals from this receptor are transmitted via a G_i protein. Pertussis Toxin (PT) destroys G_i signaling pathways because the S1 subunit of the toxin is an ADP-ribosyltransferase that modifies, and inactivates, the alpha subunit of G_i proteins. PT treatment has therefore been used to identify G_i-dependent pathways in many cell types, but its use in examining T cell signaling is complicated by the mitogenic properties of the toxin's B (non-catalytic) oligomer. To clarify the role of G_i proteins in TCR/CD3 signaling and to investigate other possible functions of G_i in T cells, we have expressed the S1 subunit of PT in the thymocytes of transgenic mice using the lymphocyte-specific *lck* promoter. S1 activity was easily demonstrated in thymocyte cell lysates of three transgenic lines. The thymuses of these mice were smaller than normal, and contained an elevated proportion of mature T cells. These mature thymic T cells responded to CD3 stimulation with normal Ca^{2+} mobilization and IL2 secretion, strongly suggesting that G_i proteins are not involved in signal transduction through CD3 or CD3-associated activation of PLC. The peripheral lymphoid organs contained very few T cells however, and this finding, together with the altered thymic phenotype, suggests that G_i proteins participate in some aspect of T cell differentiation unrelated to CD3 signalling.

F 106 ALTERED SIGNAL TRANSDUCTION IN PDGF STIMULATED RAS TRANSFORMED FIBROBLASTS. Steve R. Coats and W. J. Pledger, Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232. We have transfected Balb/c-3T3 fibroblasts with either a EJ-ras construct or a plasmid containing the insert for c-ras. Other investigators have shown that EJ-ras transformed fibroblasts while expressing PDGF receptors, exhibit a marked decrease in inositol phosphate (IP) turnover when stimulated with PDGF. To further investigate this phenomena we examined different components of this second messenger system in our ras transformed fibroblasts. As compared to normal Balb/c-3T3 fibroblasts we observed an 80% and 35% reduction in PDGF binding to its receptor in the EJ-ras and c-ras transformed cells, respectively. The EJ-ras transformed cells exhibited a total loss of IP turnover upon PDGF stimulation while the c-ras cells had a normal increase in IP turnover when stimulated with PDGF. Both the EJ-ras and the c-ras transformed fibroblast had normal down regulation of the PDGF receptor when stimulated with PDGF at 37 degrees. The EJ-ras transformed cells showed decreased levels of PDGF-dependent autophosphorylation of the PDGF receptor, while the c-ras transformed fibroblasts have a constituitively phosphorylated receptor independent of PDGF stimulation. *In vivo* analysis of Phospholipase C gamma (PLC-δ) phosphorylation showed that in the EJ-ras transformed cells the phosphorylation of PLC-δ was PDGF dependent, while in the c-ras transformed cells PLC-δ was constituitively phosphorylated. It appears that phosphorylation of the PDGF receptor and PLC-γ is not sufficient in the c-ras transformed cells to stimulate IP metabolism, as the c-ras transformed cells required the presence of PDGF for IP turnover. Anti-phosphotyrosine antibodies are being used to determine if the phosphorylations observed on PLC-$\delta$and the PDGF receptor are tyrosine specific.

F 107 MULTIPLE SECOND MESSENGER PATHWAYS OF α-ADRENERGIC RECEPTOR SUBTYPES EXPRESSED IN EUKARYOTIC CELLS. S. Cotecchia, D.A. Schwinn, J.W. Regan, M.G. Caron, R.J. Lefkowitz, HHMI, Duke Univ. Medical Center, Durham, NC

The α-adrenergic receptors (AR) mediate the effects of catecholamines on cellular signalling systems via guanine nucleotide binding regulatory proteins (G-proteins). Recent molecular cloning studies have revealed that the α-AR are a family of at least four gene products, two alpha$_1$ (α_{1A} and α_{1B}) and two alpha$_2$ (α_2 C4 and α_2 C10) AR subtypes. These receptors are structurally and pharmacologically different. Expression studies in eukaryotic cells of each α-AR subtype have indicated that both α_2-C10 and α_2-C4 couple primarily to the inhibition of adenylyl cyclase and to a lesser extent to the stimulation of polyphosphoinositide hydrolysis. Measurement of adenylyl cyclase activity in isolated cell membranes revealed that α_2-C10 inhibits adenylyl cyclase more efficiently than α_2-C4. The effects of the α_2-AR on adenylyl cyclase inhibition and on polyphosphoinositide hydrolysis are both mediated by pertussis toxin sensitive G-proteins. The major coupling system of the α_1-adrenergic receptor is to the activation of phopholipase C via a pertussis toxin insensitive G-protein. α_1-Adrenergic receptor stimulation can also increase intracellular cAMP by a mechanism that does not involve direct activation of adenylyl cyclase. These results indicate that, even though different receptor subtypes are functionally specialized in their interaction with distinct G-proteins, they can also activate multiple second messenger systems.

F 108 ANGIOTENSIN II CAUSES PHOSPHATIDYLINOSITOL TURNOVER, CA2+ SIGNALLING AND 1,2-DIACYLGLYCEROL ACCUMULATION BUT IS NOT MITOGENIC IN RAT LIVER T51B CELLS, Nicholas M. Dean and Alton L. Boynton, Basic Science Program, Cancer Center of Hawaii, 1236 Lauhala St., Honolulu, HI 96813.
The "mas" oncogene has recently been identified as being a functional angiotensin receptor, raising the possibility that in addition to a role in electrolyte balence and cardiac function angiotensin might also function as a regulator of cell proliferation. Consequently we have determined whether angiotensin II is a mitogen in rat liver T51B cells and we have examined which phospholipid-derived second messengers are generated by this hormone. Both 20% serum and 50ng/ml EGF are potent mitogens in T51B cells, however angiotensin II at no concentrations tested up to 10uM was able to increase DNA-synthesis. T51B cells contain functioning angiotensin receptors as we have found that addition of the hormone will raise intracellular Ca2+, cause an increase in phosphatidylinositol turnover and generate a sustained elevation in 1,2-diacylglycerol concentration. We were however, unable to find any changes in phosphatidylinositol(3)phosphate or an increase in the release of any choline metabolites. Our results demonstrate that the coupling of a receptor to phospholipase-C and the generation of the consequent signals is not by itself sufficient to promote DNA-synthesis.

F 109 PURIFICATION AND CHARACTERIZATION OF AN 1-O-ALKYL-2-ARACHIDONYL-PHOSPHATIDYLCHOLINE (1-O-ALKYL-2-AA-PC) HYDROLYTIC PHOSPHOLIPASE A_2 (PLA_2) FROM HUMAN MONOCYTIC LEUKEMIC U-937 CELLS. Emilio Diez and Seymour Mong. Department of Immunology, SK&F Laboratories, King of Prussia, Pennsylvania, 19406-0939
The existence of an intracellular PLA_2 for the synthesis of 1-O-alkyl-lyso-phosphatidylcholine (lyso-PAF) and free arachidonic acid (AA) has been postulated. Several investigators have tried to address this issue by purifying such an enzyme, yet, it is still unclear that any of the several purified PLA_2's are the candidate enzyme in the hydrolysis of 1-O-alkyl-2-AA-PC. Using radiolabeled 1-O-alkyl-2-AA-PC as a substrate, we have used a series of conventional and high-pressure liquid column chromatographic techniques and purified a PLA_2 from human monocytic U-937 cell cytosol to homogeneity. The purified PLA_2 has a molecular weight of 60 KD under reducing conditions by SDS-PAGE analysis. The pH optimum for enzymatic activity occurred at 8.5, and the activity is calcium dependent. A minimum of 100 nM calcium is required, and the enzyme activity increased almost 10 fold at 1 mM calcium. Under these conditions, this enzyme can hydrolyze 1-O-alkyl-2-AA-PC and 1-stearoyl-2-AA-PC; however, the activity decreased dramatically when the sn-2 position of the substrate was replaced by fatty acids other than AA. These experimental observations suggest that the 1-O-alkyl-PLA_2 could be the postulated intracellular PLA_2 responsible for the concomitant production of AA and lyso-PAF in human monocytes.

F 110 ACTIVATED p21-ras PROTEINS INHIBIT PDGF MITOGENIC SIGNAL TRANSDUCTION AND PDGF RECEPTOR AUTOPHOSPHORYLATION, Douglas V. Faller, James B. Rake, Laura Mundschau, and Michael Quinones, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115
The ras gene product has been suggested as a component of a growth factor signal transduction pathway. We have shown that the activated form of the ras p21 protein abrogates growth factor requirements for a variety of protein growth factors in their cognate cells. Furthermore, the presence of the ras protein inhibits growth factor signal transduction at an early point in the pathway. Stimulation of fibroblasts containing v-ras with PDGF-BB does not result in normally-asssociated inositol phosphate hydrolysis or the induction of growth-related gene transcription or mRNA stabilization (for c-myc, c-fos, c-jun, JE). PKC, phospholipase C and poly I:C remain able to induce these genes in the presence of v-ras. The beta-type PDGF receptor is present on these v-ras containing cells and is capable of binding PDGF-BB with normal affinity, but tyrosine phosphorylation of this receptor subunit is blocked. The blocking of PDGF receptor autophosphorylation by p21 ras is transdominant and reversible, and a membrane-associated factor is responsible. Phenotypic revertants of the v-ras-expressing cells (induced by either gene transfer or chemical treatment) demonstrate restoration of an intact PDGF signal transduction pathway.

F 111 PURINERGIC RECEPTOR AGONISTS STIMULATE CA^{2+} MOBILIZATION IN HUMAN PROSTATE CARCINOMA CELL LINES, W.-G. Fang, C.E. Myers, and J.B Trepel, Medicine Branch, NCI, NIH, Bethesda, MD 20892
Hormone-stimulated Ca^{2+} transients have not been described in prostate cells of benign or neoplastic origin. In studying the hormone responsiveness of two androgen-independent human prostatic carcinoma cell lines we found a rapid transient increase in cytoplasmic free Ca^{2+} ($[Ca^{2+}]i$) in response to purinergic receptor activation. The order of purinergic agonist potency was consistent with purinoceptors of the P_{2y} subtype. Treatment with a maximal (100 μM) concentration of ATP desensitized the receptor to subsequent purinergic activation. Chelation of extracellular Ca^{2+} decreased the peak magnitude of the Ca^{2+} transient approximately 50%. Equivalent data were obtained with dihydropyridine Ca^{2+} channel blockers and with Diltiazem. Verapamil was less active and the Ca^{2+} channel toxin ω-conotoxin was inactive. HPLC separation of inositol phosphates isolated from cells treated for 8 sec with 100 μM ATP showed an ATP-stimulated increase in IP_3. The ATP-stimulated Ca^{2+} response was unchanged in isotonic medium containing 50 mM K^+ and in medium in which Na^+ was replaced by choline, suggesting that the Ca^{2+} response to ATP is not dependent on a resting membrane potential. The ATP response was inhibited by pretreatment with cholera toxin and to a lesser extent pertussis toxin. Flow cytometric analysis of the Ca^{2+} response at the single cell level demonstrated marked heterogeneity of purinergic responsiveness within these clonal cell lines. These data demonstrate that androgen independent prostate carcinoma cells express purinergic receptors coupled to phospholipase C, potentially through a cholera toxin sensitive G protein. The data suggest that activation of this receptor opens second messenger-activated dihydropyridine-sensitive Ca^{2+} channels, prolonging the purinergic Ca^{2+} response.

F 112 CHARACTERIZATION OF THE MURINE PANCREATIC RECEPTOR FOR GASTRIN RELEASING PEPTIDE. **Bradford O. Fanger and Arlene C. Wade**, Merrell Dow Research Institute, Cincinnati, OH 45215-6300.

The murine pancreatic receptor for bombesin and gastrin releasing peptide (GRP) has been characterized. Analysis of the binding of ^{125}I-GRP to membranes indicates a single class of sites (~ 100 fmol/mg protein) with an affinity of 43 pM. ^{125}I-GRP was cross-linked to a 70 kDa membrane protein with *bis*(sulfosuccinimidyl)suberate; this labeling was completely blocked by GRP, GRP(14-17), AcGRP(20-27), neuromedin C, bombesin, and ranatensin, partially blocked by [Leu$^{13}\psi$(CH$_2$NH)Leu^{14}]bombesin, but was unaffected by GRP(21-27) and GRP(1-16). This pattern of competition was identical to that observed in binding studies with intact membranes, strongly indicating that the 70 kDa protein is the GRP receptor. In addition, the GRP receptor appears to be associated with a G-protein, since divalent cations are required for high affinity binding and nonhydrolyzable GTP analogs decrease the affinity of the receptor with little change in receptor number. In minced pancreas, GRP increased inositol phosphates by 750% in a dose-dependent manner, implicating phospholipase C in signal transduction. The findings of this study indicate that the murine pancreatic receptor for bombesin/GRP is a 70 kDa membrane protein associated with a G-protein that stimulates phosphatidylinositol turnover.

F 113 SOLUBILIZATION AND PURIFICATION OF THE GRP RECEPTOR IN AN ACTIVE FORM, Richard I. Feldman, James M. Wu, Elaina Mann and James C. Jenson, Triton Biosciences Inc., 1501 Harbor Bay Pkw., Alameda CA 94501.

The GRP receptor was purified to homogeneity from Swiss 3T3 fibroblast membranes. The receptor was solubilized by the zwitterionic detergent CHAPS in a form that maintained its ability to bind GRP. The affinity of the soluble receptor for GRP (K_D = 0.01 nM) was about the same as that measured for the receptor in intact membranes (K_D = 0.03 nM). In addition, ^{125}I-GRP binding to the soluble receptor was competed by a series of GRP-related peptides with potencies similar to those observed with the membrane bound form of the receptor. The ligand affinity of GRP receptor in membranes is reduced in the presence of GTP, GDP, GMPPNP, but not adenyl nucleotides. Guanyl nucleotides were found to increase the rate of ^{125}I-GRP dissociation from the receptor about 10-fold. In contrast, the ligand affinity of the receptor in solubilized extracts was unaffected by guanyl nucleotides indicating that it has lost the ability to couple to G proteins. The GRP receptor was purified to homogeneity by a combination of wheat germ agglutinin affinity chromatography and ligand affinity chromatography. The purified protein also exhibited similar binding affinities to GRP and related peptides as the receptor in isolated membranes. SDS PAGE analysis of the purified protein showed the presence of a single diffuse band, characteristic of a glycoprotein, with an apparent molecular weight of about 75-100 KDa. The migration of the receptor on SDS PAGE is in agreement with the value obtained by labeling the receptor in whole Swiss 3T3 cells, isolated membranes, or solubilized extracts by crosslinking to ^{125}I-GRP.

F 114 REGULATION AND FUNCTION OF GLYCOSYL-PHOSPHATIDYLINOSITOL MOLECULES IN HORMONE SIGNALING. Glen N. Gaulton and John Pawlowski. Department of Pathology and Lab Medicine, University of Pennsylvania, Philadelphia, PA 19104.

Glycosylated-phosphatidylinositol molecules (gly-PI) serve important dual functions in eukaryotic cells as membrane anchors of covalently attached proteins and as precursors to hormone-sensitive inositol-phosphoglycans that have been proposed as second messengers of hormone action. In T lymphocytes the synthesis of gly-PI is coupled to the induction of insulin receptors during early activation. Insulin also stimulates the rapid hydrolysis of 56% of cellular gly-PI. The resultant IP-gly is a potent regulator of several enzymes including cAMP-dependent kinase and cAMP-phosphodiesterase. To further investigate the structure and function of these molecules monoclonal antibodies were constructed in mice and were screened by both RIA and HPTLC overlay for specific reactivity to the glycan moiety of gly-PI. These techniques were successful in the detection of four gly-PI subtypes in T cells. Each of these subtypes is sensitive to both phospholipase C and deamination by nitrous acid. However, only two of these subtypes were hydrolyzed following insulin binding. The heterogeneity in insulin responsiveness and structure of gly-PI may relate to unique functions of gly-PI as protein anchors and signal transduction elements. Antibodies to gly-PI have also been used to label intact and permeabilized lymphocytes as a means to identify the subcellular localization of these molecules. Prominent staining was detected on the cell surface, golgi and nuclear envelope.

F 115 PERSISTENT INDUCTION OF CYCLOOXYGENASE IN $p60^{v\text{-}src}$ TRANSFORMED 3T3 FIBROBLASTS, Jia-wen Han, Henry Sadowski, Donald A. Young and Ian G. Macara, Environmental Health Sciences Center, Departments of Biophysics and Medicine, University of Rochester Medical Center, Rochester, NY 14642

A balb/c 3T3 cell-line infected with the temperature-sensitive Rous sarcoma virus LA 90 has been used to investigate early changes in gene expression (protein synthesis) after activation of $p60^{v\text{-}src}$. By using rabbit polyclonal antiserum against sheep seminal vesicle cyclooxygenase, which cross-reacts with mouse cyclooxygenase, it was found that the synthesis of cyclooxygenase [prostaglandin (PG) synthase or PG endoperoxide synthase] was markedly stimulated by the activation of the temperature-sensitive $p60^{v\text{-}src}$. The induction of cyclooxygenase by $p60^{v\text{-}src}$ was rapid and persistent. In contrast, platelet-derived growth factor (PDGF) induced only a transient increased synthesis of cyclooxygenase, which returned to the baseline levels within 8-12 hours of stimulation. The kinetics of changes in cellular cyclooxygenase activity stimulated by $p60^{v\text{-}src}$ or PDGF was paralleled closely by changes in the abundance of a 74-78 kilodalton protein doublet labeled with [^{35}S]methionine that could be immunoprecipated by the anti-cyclooxygenase antiserum. Down regulation of protein kinase C by chronic administration of phorbol ester did not block the induction of cyclooxygenase in $p60^{v\text{-}src}$ transformed LA 90 cells. The antiinflammatory glucocorticosteroids dexamethasone (DEX) inhibited the induction of cyclooxygenase by $p60^{v\text{-}src}$. This report indicates that oncogenic protein $p60^{v\text{-}src}$ persistently enhanced the synthesis and cellular enzymatic activity of cyclooxygenase in 3T3 fibroblasts with different kinetics of induction than the growth factor, PDGF.

F 116 **PLC-γII: cDNA CLONING, EXPRESSION, PURIFICATION AND CHARACTERIZATION.** Homma, Y., Emori, Y.*, Suzuki, K.*, and Takenawa, T. Tokyo Metropolitan Institute of Gerontology and *Tokyo Metropolitan Institute of Medical Science, Japan

PLC-γII has been cloned for cDNA by screening a rat muscle cDNA library with cDNA probes of previously identified PLC species. PLC-γII, in common with PLC-γ (-γI), has two homologous domains (X and Y), and a sequence homologous to the N-terminal regulatory domains of nonreceptor tyrosine kinases of the src-family of oncogenes (Z domain). Using an E. coli expression system, PLC activity has been recovered in the crude extract. Various truncation experiments of PLC-γII cDNA reveals that the src-related Z domain is not necessary for catalytic activity while both X and Y domains are essential. PLC-γII mRNA is expressed in various rat tissues and abundant in spleen, lung and thymus. To characterize this enzyme, PLC-γII protein has been purified to apparent homogeneity from a crude extract of bovine spleen. The purified enzyme has a molecular mass of 145 kDa on SDS polyacrylamide gel electrophoresis and a specific activity of 25.3 μmol/min/mg protein. It hydrolyzes three inositol-phospholipids in a Ca^{2+}- and pH-dependent manner.

F 117 MICROINJECTED ANTIBODIES TO PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE (PIP2) BLOCK Ca^{2+} RESPONSE TO PDGF IN SINGLE VASCULAR SMOOTH CELLS.

Harlan E. Ives, Kiyoko Fukami, Tadaomi Takenawa and Chou-Long Huang. Cardiovascular Research Inst. U.C. San Francisco, S.F., CA; Tokyo Metropolitan Inst. of Gerontology, Japan.

Activation of phospholipase C by PDGF causes breakdown of PIP2 with release of inositol 1,4,5-trisphosphate (IP3), the second messenger that mobilizes internal Ca^{2+} stores. PDGF mobilizes Ca^{2+} much more slowly (1-2 min.) than other agonists which release IP3 (e.g., thrombin mobilizes Ca^{2+} over 15s). We therefore sought to determine whether mobilization of Ca^{2+} by PDGF is entirely via PIP2 metabolites. Intracellular free Ca^{2+} (iCa^{2+}) was measured in single vascular smooth muscle cells using fura2 microspectrofluorometry. PDGF (recombinant BB, 10 ng/ml) raised iCa^{2+} from 112 ± 20 nM to 650 ± 70 nM after an average 129 sec delay. In medium containing 2mM Ca^{2+}, iCa^{2+} remained elevated for > 10 min. after PDGF. In nominally Ca^{2+}-free medium, iCa^{2+} fell to baseline after < 5min exposure to PDGF; readdition of 2 mM Ca^{2+} to the medium after 5 min. caused a second increase in iCa^{2+} similar in magnitude to the first. Thus PDGF appears to mobilize Ca^{2+} from both internal and external stores. Microinjection of monoclonal PIP2 Ab (kt10, 2 mg/ml) totally abolished both phases of PDGF-induced iCa^{2+} increase in 6 out of 9 cells examined. Neither unimmunized mouse nor bovine IgG affected the Ca^{2+} response to PDGF (0 out of 10 cells), nor did monoclonal Ab against protein kinase C (0 out of 5 cells). We conclude that elevation of iCa^{2+} by PDGF occurs both by mobilization of internal Ca^{2+} stores and by extracellular uptake and that both phases depend upon second messengers generated from PIP2.

F 118 BRADYKININ STIMULATED RELEASE OF EICOSANOIDS FROM RAS-TRANSFORMED FIBROBLASTS.

Gordon A. Jamieson, Jr., Kimberly A. Flem, Hussein A. Elkousy, Scott A. Holliday and George D. Leikauf, Division of Pulmonary Cell Biology, Department of Environmental Health, Univ. Cincinnati Med Center, Cinti, OH 45267.

Previously we determined serum to rapidly stimulate arachidonic acid release (AAR) from H-ras or K-ras transformed cells, but not from parental NIH/3T3 cells. Ras-transformation has been demonstrated by others to increase the expression of bradykinin receptors. Consequently we hypothesized the increase in AAR we observed upon ras-transformation might be due to altered expression of a bradykinin-coupled phospholipase A2 (PLA2) activity. Hence we tested the ability of bradykinin and the Ca ionophore A23187 to stimulate AAR in normal and ras-transformed cells and analyzed for the formation of specific eicosanoids by HPLC. The predominant eicosanoid (>95% [3H]-material) formed upon bradykinin stimulation of K-ras transformants was determined to be PGE2, an eicosanoid with growth regulatory properties. ELISA confirmed the identification of the released product to be PGE2. A23187 stimulated greater release of [3H]-material in ras-transformed cells (4-6 fold) than in the parental NIH/3T3 cells, suggesting increased expression of PLA2 upon H-ras or K-ras transformation. In contrast, bradykinin stimulated the release of arachidonate from the K-ras transformants but not from the H-ras transformants, implying that the bradykinin receptor may be coupled to PLA2 only in the K-ras transformed NIH/3T3 cells. These results are consistent with the concept that ras-transformation can alter PLA2 levels, bradykinin receptor-PLA2 coupling and subsequent generation of growth regulatory eicosanoids.

Supported by University Research Council, NIH SO7-RR05408 and NIEHS ES 00159.

F 119 MUSCARINIC RECEPTORS MEDIATE RELEASE OF ARACHIDONIC ACID IN SPINAL CORD NEURONS INDEPENDENT OF INOSITOLPHOSPHOLIPID HYDROLYSIS.

Robert Y. Kanterman*, Christian C. Felder,and Julius Axelrod, Laboratory of Cell Biology, National Institute of Mental Health, Bethesda, MD, 20814; *Howard Hughes Medical Institute-NIH Research Scholars Program.

Receptor-mediated release of arachidonic acid in the CNS has recently been demonstrated in primary cultures of striatum, cerebellum, hippocampus and cortical astrocytes. We have previously demonstrated that α_1-adrenergic receptors mediate the release of arachidonic acid in spinal cord neurons independent of inositolphospholipid hydrolysis (in press, *J. Neurochem.*). In mixed neural-glial cultures of spinal cord cells, the acetylcholine agonist carbachol stimulated both release of arachidonic acid and turnover of inositolphospholipids via muscarinic receptors. Carbachol failed to cause the release of arachidonic acid from spinal cord glia, indicating that the carbachol-induced release of arachidonic acid was neuronal in origin. Phorbol ester (PMA) inhibited the production of inositolphosphates but not release of arachidonic acid consistent with other examples of receptor-mediated activation of phospholipase A_2. Additionally, carbachol stimulated the release of arachidonic acid in hippocampal neurons and cerebral cortical neurons (but not cerebellar granule cells) in primary culture. Preliminary results suggest that muscarinic receptors mediate the release of arachidonic acid via the phospholipase A_2 transmembrane signalling pathway in several tissues in the CNS.

F 120 PRIMING OF EOSINOPHILS INDUCES THE FORMATION OF ETHERLINKED DIGLYCERIDES UPON ACTIVATION WITH OPSONIZED PARTICLES.

Leo Koenderman, Anton Tool, Taco Kuijpers, Dirk Roos and Arthur Verhoeven. Central Lab. Neth. Red Cross Blood Trans. Service and Lab. Exp. Clin. Immunology, University of Amsterdam, Amsterdam, The Netherlands.

Addition of STZ (yeast particles opsonized with IgG and the complement factor iC3b) to human eosinophils results in a rather low cell activation as measured by respiratory burst activation and homotypic aggregation. These eosinophil responses are markedly enhanced after pretreatment with platelet activating factor (PAF). We investigated whether this "priming" with PAF causes a change in accumulation of second messengers. Addition of STZ to control cells leads to a late and moderate accumulation of diradylglycerols with no increase of $[Ca^{2+}]_i$, which illustrates the minor importance of hydrolysis of PIP2 under these circumstances. The diradylglycerol species that accumulates is mainly of the diacylglycerol type (DAG). After priming with PAF, the mass of diradylglycerols that accumulates in response to opsonized particles is markedly enhanced, and this enhancement is due to the accumulation of both 1,2-diacylglycerol and ether-linked diglycerides (EAG). Moreover, addition of opsonized particles to these PAF-treated cells is accompanied by a rise in $[Ca^{2+}]_i$, which is in part of extracellular origin. These findings indicate that PAF priming of eosinophils profoundly changes the coupling of the opsonin receptors to intracellular phospholipases.

F 121 FORMATION OF 3-PHOSPHORYLATED PHOSPHOINOSITIDES IN STIMULATED HUMAN PLATELETS, Gregory L. Kucera and Susan E. Rittenhouse, Department of Biochemistry, University of Vermont, Burlington, VT 05405. Human platelets stimulated with α-thrombin or the thromboxane A_2 analogue U-46619 form the novel phosphatidylinositol polyphosphate species phosphatidylinositol-3,4-bisphosphate (PtdIns3,4P_2) and phosphatidylinositol-3,4,5-trisphosphate (PtdIns3,4,5P_3) in a time and dose-dependent manner. In [^{32}P] labeled platelets, PtdIns3,4P_2 increases in 120 seconds to 508% of the basal level with 1 U/ml α-thrombin, while 5 μM U-46619 stimulates the production of this isomer of PtdInsP_2 by 339%. PtdIns3,4,5P_3 is also increased with α-thrombin and U-46619 stimulation by 210 and 163%, respectively. PtdIns3P quantities remain nearly equal to those under resting conditions. The basal or increased levels of PtdIns3,4P_2 do not appear to be adequate to account for the rapid elevation of Ins1,3,4P_3 observed in thrombin-stimulated platelets.

A23187 and/or phorbol dibutyrate are relatively unsuccessful at stimulating changes in PtdIns3,4P_2 and PtdIns3,4,5P_3 compared with U-46619 or α-thrombin. This indicates that Ca^{2+} and protein kinase C, following phospholipase C activation, are not sufficient to promote the formation of these unusual phosphatidylinositol polyphosphates. GTPγS (100 μM), however, increases the level of PtdIns3,4P_2 by 2274% and that of PtdIns3,4,5P_3 by 184% in saponin-permeabilized platelets incubated with 0.5 mM [^{32}P]γATP, implying a role for a GTP-binding protein in promoting phosphatidylinositide 3-kinase activity (ies).

This is the first report of stimulated generation of 3-phosphorylated phosphoinositides in anucleate cells. A role for these novel phospholipid species in signal transduction, however, awaits elucidation.

F 122 PDGF INDUCES TYROSINE-PHOSPHORYLATION OF THE REGULATORY (SH2/SH3) DOMAIN OF PLC-γ1,AND INCREASED PLC-γ1 ACTIVITY,Lih-Ling Lin[1],Lisa Sultzman[1],Christine Ellis[2],Tony Pawson[2],and John Knopf[1]. [1]Genetics Institute,Cambridge MA.,and Mount Sinai Hospital Research Institute,Toronto,Ontario.

PDGF treatment of fibroblasts results in a number of rapid responses. One of these responses is an increased breakdown of phosphatidylinositol (PI) which is mediated by phospholipase C(PLC) PLC-γ1 has recently been shown to be rapidly phosphorylated on tyrosine in response to PDGF. We have overexpressed PLC-γ1 in fibroblasts and demonstrated an increased PI breakdown in response to PDGF, but not to AlF_4^-,a universal activator of G protein coupled PLC's. This demonstrates that the increased PI breakdown noted in fibroblasts is due to the activation of PLC-γ1. Additionally we have shown that PLC-γ2, a close relative of PLC-γ1, can also be phosphorylated on tyrosine in response to PDGF treatment. We have mapped the tyrosine phosphorylation sites of PLC-γ1to the regulatory (SH_2/SH_3) domain which is shared by PLC-γ1 and PLC-γ2. Additional regulators of PLC will be presented as well as further mutational analysis.

F 123 THE ASSOCIATION OF TYPE I PHOSPHOINOSITIDE KINASE ACTIVITY WITH POLYOMA MIDDLE T/pp60$^{c\text{-}src}$ COMPLEX, †Leona E. Ling, *Leslie Serunian, *Lewis Cantley and †Thomas M. Roberts. †Dana-Farber Cancer Institute, Physiology, *Tufts University School of Medicine, Boston, MA.

The type I phosphatidylinositol kinase activity associates with polyoma middle T/pp60$^{c\text{-}src}$ transforming protein complex in polyoma infected cells and with PDGF receptor in PDGF activated smooth muscle cells. This lipid kinase phosphorylates the 3-D position of the inositol ring of phosphatidylinositol (PI). Transformation defective mutants of polyoma middle T, NG59, py1387T, dl23 and py1178T either lack or have diminished amounts of associated type I PI kinase activity whereas transformation competent wild type or mutant middle T/pp60$^{c\text{-}src}$ complex always contains high amounts of type I activity. However, there are several middle T mutations, dl1015 and py248m which abolish transformation, but still allow binding of the type I PI kinase. In lieu of the recent findings that:

1)both middle T/pp60$^{c\text{-}src}$ complex and activated PDGF receptor bind a 3-D position lipid kinase which phosphorylates PI(4)P and PI(4,5)P_2 [Serunian, L. A., Auger, K. R., Roberts, T. M. and Cantley, L. C., manuscript submitted].

2) that the products of these reactions PI(3,4)P_2 and PI(3,4,5)P_3 are elevated both in cells transformed by polyoma middle T and in cells stimulated with PDGF [Auger, K. R., Serunian, L. A., Soltoff, S. P., Libby, P. and Cantley, L.C., Cell 57, 167 (1989)].

the ability of the lipid kinase activities associated with these polyoma middle T mutants to produce PI(3,4)P_2 and PI(3,4,5)P_3 were tested *in vitro* and *in vivo*.

F 124 HUMAN GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR DIRECTLY STIMULATES PLATELET ACTIVATING FACTOR SYNTHESIS IN HUMAN NEUTROPHILS, A.F. LOPEZ, M.O. DeNichilo, J.G. Gamble, M.A. Vadas, A.G. Stewart. Div. of Human Immunology, Inst. of Medical and Veterinary Science, Adelaide, Sth.Aust. 5000 Australia, Dept. of Physiology, Uni. of Melbourne, Victoria 3000 Australia.

Human granulocyte-macrophage colony-stimulating factor (GM-CSF) directly stimulates several neutrophil functions in vitro. In addition, GM-CSF 'primes' neutrophils for enhanced biologic response to a number of secondary stimuli. The underlying mechanism by which this cytokine mediates its effect is still poorly understood. We found GM-CSF to directly stimulate the synthesis of platelet-activating factor (PAF) in purified human neutrophils, as measured by bioassay. In a time- and dose-dependent manner, with GM-CSF stimulating levels of PAF comparable with that of two other neutrophil agonists, FMLP and TNF-α. In addition, PAF synthesis was dependent on a pertussis toxin sensitive GTP-binding protein. The synthesized PAF was retained wholly within the cell, raising the possibility that PAF acts as a second messenger in the GM-CSF signal transduction pathway. To investigate the relationship between PAF production and the stimulation of neutrophil function, we employed two inhibitors of PAF synthesis, TPCK and Bromophenacyl Bromide. Our studies showed that inhibition of PAF synthesis closely paralleled inhibition of GM-CSF stimulated neutrophil adherence to human umbilical vein endothelial cells. Moreover, inhibition of stimulated adherence could also be achieved by pre-incubating neutrophils with specific PAF receptor antagonists. These results identify PAF as an intracellular product of GM-CSF stimulation, and suggest a second messenger role for PAF in GM-CSF stimulated neutrophil function.

F 125 THE ASSOCIATION OF PLC-γ WITH OTHER CELLULAR PHOSPHOPROTEINS.
Jill Meisenhelder and Tony Hunter, MBVL, Salk Institute, La Jolla, CA 92037

We have recently shown that PDGF and EGF treatment of cells leads to rapid and extensive phosphorylation of PLC-γ tyrosine (as well as serine) residues. This increased phosphorylation occurs only in cell lines in which these growth factors also stimulate PLC activity. Tryptic phosphopeptide mapping of PLC-γ from growth factor-treated cells reveals that it is phosphorylated at tyrosines identical to those seen when purified PLC-γ is incubated in vitro with purified growth factor receptors. We, as well as several other groups, have demonstrated a physical association between ligand-activated EGF and PDGF receptors and PLC-γ by coprecipitating the two molecules using a mixture of monoclonal antibodies (MAb) directed against PLC-γ. We continue to be interested in the regulation of PLC-γ activity, and have been studying three other phosphoproteins (of approximately 100, 84 and 47 kDa) that are coprecipitated with PLC-γ. Peptide mapping and immunoblotting indicate that all three proteins are largely unrelated to PLC-γ. Gradient analysis and comparison of immunoprecipitates made from fully denatured versus detergent cell extracts show that p100 and p84 are clearly associated with PLC-γ, but the reason why p47 coprecipitates remains unclear. Unlike the PDGF receptor, the association of these proteins does not change with ligand stimulation. Analysis using individual MAbs shows that only a small subpopulation of PLC-γ is associated with these three proteins. Details of the phosphorylation state of this complexed PLC-γ, and further experiments on the nature of the interactions between these proteins and the identities of p100, p84 and p47 will be presented.

F 126 **MULTIPLE SIGNALING PATHWAYS OF HISTAMINE H_2 RECEPTORS, Masato Mitsuhashi and Donald G. Payan, Howard Hughes Medical Institute Laboratories and Department of Medicine, University of California Medical Center, San Francisco, CA 94143**

In order to analyze the complex activities of histamine H_2 receptor (H_2R) activation on neutrophils, human HL-60 promyelocytic leukemia cells were differentiated into neutrophils by incubation with dimethylsufoxide, loaded with the Ca^{2+} sensitive indicator dyes, indo-1 or fura-2, and the levels of intracellular Ca^{2+} ($[Ca^{2+}]i$) measured in a flow cytometry and fluorimeter, respectively. Histamine (HA) increased $[Ca^{2+}]i$ in a dose dependent manner with a half maximal concentration of approximately 1 μM, which exhibited H_2R specificity. Prostaglandin E_2 and isoproterenol also mobilized $[Ca^{2+}]i$ in HL-60 cells, whereas the cell permeable form of cAMP and forskolin failed to increase $[Ca^{2+}]i$. Since H_2R-mediated $[Ca^{2+}]i$ mobilization was not inhibited by reducing the concentration of extracellular Ca^{2+} nor by the addition of Ca^{2+} channel antagonists, $LaCl_3$ and nifedipine, Ca^{2+} might be released from intracellular stores. Furthermore, both 100 μM HA and 1 μM f-Met-Leu-Phe (fMLP) increased the levels of inositol trisphosphate ($1,4,5\text{-}IP_3$). However, HA-induced mobilization of $[Ca^{2+}]i$ was inhibited by cholera toxin (CT), but not by pertussis toxin (PT), whereas the action of fMLP was inhibited by PT, but not by CT. These data suggest that H_2R on HL-60 cells are coupled to two different CT sensitive G-proteins, and activate adenylate cyclase and phospholipase C simultaneously.

F 127 PDGF INDUCES RAPID TYROSINE PHOSPHORYLATION OF THE *RAS* p21 GTPase ACTIVATING PROTEIN (GAP) IN QUIESCENT NIH3T3 FIBROBLASTS, Christopher J. Molloy[1], Donald P. Bottaro[1], Timothy P. Fleming[1], Mark S. Marshall[2], Jackson B. Gibbs[2], and Stuart A. Aaronson[1], [1]LCMB, National Cancer Institute, Building 37, Room 1E24, Bethesda, MD 20892 , and [2]Department of Molecular Biology, Merck Sharp and Dohme Research Laboratories, West Point, PA. The cascade of biochemical events triggered by growth factors and their receptors has become central to understanding normal cell growth regulation and its subversion in cancer. *Ras* proteins (*ras* p21) have been implicated in signal transduction pathways utilized by several growth factors including PDGF. These guanine-nucleotide binding proteins specifically interact with a cellular GTPase activating protein (GAP), which stimulates the GTPase activity of normal but not oncogenic forms of *ras* p21. We show that in intact, quiescent fibroblasts, both PDGF-AA and BB homodimers rapidly induced tyrosine phosphorylation of GAP under conditions in which insulin and basic fibroblast growth factor (bFGF) were ineffective. This occurred within 1 minute of growth factor addition, was maximal at 10 minutes and persisted for up to 8 hours. Although GAP was predominately localized in the cytosol, the majority of tyrosine phosphorylated GAP was associated with cell membranes, the known site of *ras* p21 biologic activity. These results indicate a direct biochemical link between activated growth factor receptor tyrosine kinases and the *ras* p21/GAP mitogenic signaling system.

F 128 Cyclic AMP agonists increase the phosphorylation of phospholipase Cγ and decrease formation of inositol phosphates in BALB/c-3T3 cells. Nancy E. Olashaw and W.J. Pledger, Vanderbilt University, Nashville, TN

Phospholipase C (PLC), a family of isozymes, mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the consequent formation of two potential second messengers, inositol 4,5-trisphosphate and diacylglycerol. Using monoclonal antibodies specific to PLCγ, we found that the cyclic AMP agonists cholera toxin and isobutylmethylxanthine (IBMX) induced the phosphorylation of PLCγ on serine and/or threonine residues in density-arrested BALB/c-3T3 cells. Increased phosphorylation of PLCγ was evident within 15 min of addition of cholera and IBMX to cells, was maximal at 30 min, remained elevated for at least 60 min and was unaffected by cycloheximide. The increase in PLCγ phosphorylation produced by cholera toxin and IBMX was accompanied by changes in the pattern of phosphoproteins that co-precipitated with PLCγ. When added to cells at the same time as or 15 or 30 min before aluminum fluoride, cholera toxin and IBMX decreased aluminum fluoride-stimulated inositol phosphate formation approximately 50%. Thus, phosphorylation of PLCγ by cyclic AMP agonists correlates with inhibition of aluminum fluoride-induced inositol phosphate formation.

F 129 CALCIUM CHANNEL AGONISTS POTENTIATE TRH-STIMULATED INOSITOL PHOSPHATE PRODUCTION AND PROLACTIN SECRETION, Jonathan A. Pachter, Greg J. Law and Priscilla S. Dannies, Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510 Thyrotropin-releasing hormone (TRH) has been previously shown to stimulate prolactin secretion by elevating inositol polyphosphate and cytosolic calcium levels. When added to perifused primary cultures of anterior pituitary cells, the dihydropyridine (DHP) calcium channel agonist BAY K 8644 (1 μM) stimulated basal prolactin secretion and potentiated stimulation of prolactin secretion by TRH (1 μM) 5-fold. These effects of BAY K 8644 were mimicked by other DHP agonists, CGP 28392 (10 μM) and (+)SDZ 202-791 (10 μM), and by (-)-BAY K 8644, while (+)BAY K 8644 had no effect on basal or TRH-stimulated prolactin release. The DHP calcium channel antagonist nimodipine, at 10 μM, a concentration sufficient to block BAY K 8644-stimulated ^{45}calcium uptake, blocked the enhancement of basal and TRH-stimulated prolactin release by BAY K 8644. These results suggest that DHP agonists potentiate TRH-induced hormone release through interaction with the known stereospecific DHP receptor sites on L-type calcium channels. Although BAY K 8644 alone had no effect on inositol polyphosphate accumulation, the calcium channel agonist enhanced TRH-stimulated accumulation of inositol 1,3,4-trisphosphate and inositol 1,3,4,5-tetrakisphosphate at relatively late times (20 minutes), but did not enhance accumulation of inositol 1,4,5-trisphosphate, the active isomer in intracellular calcium mobilization. These data suggest that although BAY K 8644-induced calcium entry may stimulate inositol trisphosphate-3-kinase activity, the potentiation of TRH-stimulated hormone secretion by BAY K 8644 probably does not result from calcium-induced enhancement of inositol polyphosphate formation.

F 130 CHOLERA TOXIN INDUCES GENE EXPRESSION BY TWO DISTINGUISHABLE SIGNAL-TRANSDUCTION PATHWAYS IN BALB/c 3T3 CELLS:
Sajjad A. Qureshi, Konstantina Alexandropoulos, Rudolph Spangler, and David A. Foster. The Institute for Biomolecular Structure and Function and The Department of Biological Sciences, The Hunter College of The City University of New York, 695 Park Avenue, New York, New York 10021.

Cholera Toxin (CT) catalyzes ADP-ribosylation of a subset of G-proteins. ADP-ribosylation of the adenylyl cyclase stimulatory G-protein, G_s, leads to increases in intracellular cAMP levels. CT induces the expression of two murine immediate-early response genes (TIS10 and JE) in quiescent BALB/c-3T3 cells. Increases in cAMP levels activates TIS10 gene expression but has no effect on JE gene expression. In addition we find that 3-isobutyl-1-methylxanthine (IBMX), which stabilizes intracellular cAMP, potentiates CT-induced TIS10 gene expression but has no effect on CT-induce JE gene expression. CT-induced expression of TIS10 and JE is differentially sensitive to protein kinase inhibitors and nordihydroguaiaretic acid (NDGA), an inhibitor of lipoxygenase activity suggesting separate mechanisms for induction of TIS10 and JE by CT. Taken together, these data demostrate that CT activates two distinguishable signal-transduction pathways in BALB/c 3T3 cells: one that is dependent upon cAMP production and another that is independent of cAMP production.

F 131 SELECTIVE INHIBITION OF BOTH THE PDGF RECEPTOR TYROSINE KINASE AND PDGF-DEPENDENT PHOSPHOLIPASE C ACTIVATION BY STAUROSPORINE IN SWISS 3T3 CELLS.
J. Paul Secrist, Inder Sehgal, Richard Olsen, Garth Powis, and Robert T. Abraham. Departments of Immunology and Pharmacology, Mayo Clinic/Foundation, Rochester, MN 55905.
The binding of PDGF to specific receptors (PDGF-R) expressed on quiescent fibroblasts initiates the generation of transmembrane signals that culminate in cellular proliferation. Proximal events linked to mitogenic signal transmission from ligand-bound PDGF-R are stimulation of the receptor tyrosine kinase activity and phosphoinositide hydrolysis due to activation of phospholipase C (PLC). Recent data indicate that PLC-γ is a substrate for the PDGF-R tyrosine kinase; however, the relationship between tyrosine phosphorylation on PLC-γ and PLC activation by PDGF remains unclear. In this study, we demonstrate that the protein kinase inhibitor, staurosporine, is a potent inhibitor of the PDGF-R tyrosine kinase activity in Swiss mouse 3T3 fibroblasts. Staurosporine (0.1-1 μM) pretreatment inhibits PDGF-dependent tyrosine phosphorylation of both the PDGF-R and PLC-γ. Furthermore, staurosporine concomitantly blocks PDGF-dependent PLC activation and the subsequent increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). In contrast, the PLC activation and $[Ca^{2+}]_i$ increase induced by bradykinin, which do not involve tyrosine kinase activation, are staurosporine-insensitive. Thus, staurosporine is a selective inhibitor of both the phosphorylation of PLC on tyrosine residues and the stimulation of phosphoinositide hydrolysis induced by ligand-bound PDGF-R . These data strongly suggest a causal relationship between PLC-γ phosphorylation and PLC activation in PDGF-stimulated fibroblasts. Supported by CA42286 (GP) and DCB-8616001 (RTA).

F 132 RELATION OF CYTOPLASMIC FREE CALCIUM CONCENTRATION (Ca_i), DIACYLGLYCEROL (DAG), AND INOSITOL PHOSPHATE SIGNALS TO HUMAN NEUTROPHIL SUPEROXIDE (O_2^-) PRODUCTION.
GB Segel, S Liang, TJ Woodlock, J Whitin, and MA Lichtman, University of Rochester School of Medicine, Rochester, NY. The time course and magnitude of change in three intracellular messengers were determined in relation to FMLP (1 uM) induced O_2^- production. The temporal sequence involved a peak rise in Ca_i, followed by O_2^- generation, followed by an increment in DAG mass. In the presence of 1 mM EGTA, the FMLP-induced increment in Ca_i was reduced by 75%, O_2^- production was reduced by 50% ($P < 0.05$), and there was no change in the FMLP-induced increment in DAG mass. Preincubation with 1 ug/ml pertussis toxin (PT) for 1 hr decreased FMLP-induced O_2^- production by 52%, suggesting a role for G-proteins in the FMLP response. The FMLP-induced increments in Ca_i and DAG mass were not altered by such PT treatment although higher PT concentrations or longer exposure times did decrease the DAG increment. Moreover, 1 ug/ml PT caused an 81% decrease in FMLP-induced inositol phosphate generation, suggesting that PT inhibits the phospholipase responsible for hydrolysis of phosphatidylinositol-bis-phosphate (PIP_2) and that DAG generation from PIP_2 is decreased. Upon neutrophil stimulation with 0.5 uM ionomycin, there was no O_2^- production although ionomycin caused a marked increase in Ca_i and in DAG mass. Taken together, these data suggest that: 1) an increment in Ca_i is necessary but not sufficient to initiate O_2^- production in response to FMLP, 2) an increment in DAG mass does not precede the initiation of O_2^- production by FMLP, and 3) PT inhibits FMLP-induced O_2^- production by blocking the formation of inositol phosphates and DAG generated from PIP_2.

F 133 GLYCOSYL-PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE D (GPI-PLD): A POSSIBLE CONTRIBUTOR TO SERUM-INDUCED MITOGENESIS IN NIH 3T3 FIBROBLASTS. Ann Sesko & Martin G. Low. Dept. of Physiology & Cellular Biophysics, College of Physicians & Surgeons of Columbia University, New York 10032. GPI-PLD is a serum-derived enzyme which displays activity toward GPI-anchored proteins on the surfaces of cells. Assessed in vitro by the hydrolysis of [^{3}H]myristate-labeled substrate (i.e. variant surface glycoprotein of Trypanosoma brucei, VSG), to produce butanol-soluble phosphatidic acid and water-soluble protein, the activity of GPI-PLD in vivo has not been demonstrated directly. Work of others has shown that phosphatidic acid or bacterial phospholipase D, like fresh serum, are mitogenic when added to the culture medium of serum-deprived 3T3 cells. In order to determine whether mammalian GPI-PLD contributes to this mitogenesis, quiescent NIH 3T3 cells were stimulated with a variety of agents including: i) serum, ii) serum depleted of GPI-PLD by immunoaffinity chromatography, iii) depleted medium supplemented with purified GPI-PLD, iv) depleted medium supplemented with PLD from S.chromofuscus, or v) phosphatidic acids of various fatty acyl compositions, and the growth fraction of cultures was assessed. Multiple dosages of each agent were employed and the activity of GPI-PLD in depleted and undepleted medium was determined by hydrolysis of [^{3}H]VSG. Mitogenesis was quantified either by incorporation of [^{3}H] thymidine into acid-precipitable material or by counting of cells. Preliminary data indicates that media containing approximately 20% of control levels of GPI-PLD are no less mitogenic as than those containing whole serum. Results of these and studies with further reductions in GPI-PLD levels will be presented. Supported by NIH grants #HL07114-15 and GM40083-01A1.

F 134 CHARATERIZATION AND ISOLATION OF THE MSH-RECEPTOR OF B16 MELANOMA, Flavio F.A. Solca, Walter Siegrist, Roma Drozdz, Jürg Girard and Alex N. Eberle, Department of research (ZLF), University hospital, CH-4031 Basel, Switzerland. The melanotropin receptor of mouse B16-F1 melanoma cells was characterized by photoaffinity crosslinking, using a potent α-MSH photolabel, [norleucine^4, D-phenylalanine^7, 1'-(2-nitro-4-azido phenyl-sulfenyl)-tryptophan^9]-α-MSH (Naps-MSH). Its monoiodinated form, ^{125}I-Naps-MSH, displayed a ~6.5-fold higher biological activity than α-MSH. Scatchard analysis of the saturation curves with ^{125}I-Naps-MSH revealed ~20,000 receptors/B16-F1 cell and an apparent K_D of ~0.3 nM. Analysis of the crosslinked MSH receptor by SDS-polyacrylamide gel electrophoresis showed that a photolabeled band of approximately 45 kDa occurs in B16-F1, B16-F10 and Cloudman S91 mouse melanoma as well as in human D10 and 205 melanoma but not in non-melanoma cells. The labeled 45 kDa protein had an isoelectric point of 4.5-4.9 as determined by two-dimensional gel electrophoresis. Treatment of the labeled 45 kDa protein of B16-F1 cell membranes by neuraminidase shifted the band to approximately 42 kDa. A similar band of about 42 kDa was also observed after receptor labeling of B16-W4 cells, a cell line with a decreased number of terminal *N*-linked neuraminyl residues. These results indicate that the labeled 45 kDa glycoprotein contains terminal sialic acid residues, explaining the low pI of this protein, and that it is characteristic for melanoma cells and hence part of the MSH receptor.

F 135 **A RAS ONCOGENE SUPPRESSOR FUNCTIONS BY REGULATING SIGNAL TRANSDUCTION THROUGH** MULTIPLE GROWTH FACTOR RECEPTORS. Michael A. Tainsky, Paul Chiao, and Sun O. Yim, DEPT. OF TUMOR BIOLOGY, UNIV. OF TEXAS, M.D. ANDERSON HOSPITAL CANCER CENTER HOUSTON, TEXAS

A human cell culture system has been developed to identify how oncogenes' action is regulated during the of transitions in multistage carcinogenesis. PA-1 human teratocarcinoma cells show progression as they are passaged in culture. At passage 30 they have reverted and are nontumorigenic in nude mice. Certain preneoplastic clonal cell lines in this PA-1 series are susceptible to transformation by single (ras) oncogenes while others require both myc and ras oncogenes to induce tumorigenesis. Based on experiments with somatic cell hybrids, the basis of this susceptibility to single oncogene induced transformation appears to be due to loss of a suppressor gene. Myc must therefore by-pass the regulatory effects of the suppressor gene. We have found that the mechanism by which cells acquire the susceptibility to ras is related to responsiveness to epidermal growth factor, basic-fibroblast growth factor and transforming growth factor-alpha possibly by affecting an autocrine mechanism. The suppressor may function to regulate signal transduction through multiple receptors. This represents a new role of suppressor gene inactivation during a preneoplastic stage progressing toward tumorigenicity. Differences in expression of growth factor responsive genes among these cells will be discussed.

F 136 SIGNAL TRANSDUCTION OF INTERFERONS, Y.H. Tan, Yap Wai Ho, Andrew Lim, Sim Tse'fong and S.D. Menon, Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511. Transient increases in the concentration of cellular diacylglycerol are observed in a variety of mammalian cell types in response to interferon treatment. These increases vary from 0.1 to 2.5 fold and are extremely rapid, lasting for about 30 seconds. Small increases in the activity of protein kinase C are also observed in interferon-treated cells, occurring after the rapid increases in diacylglycerol and lasting for about 5 min. These increases are variable with several experimental parameters. Diacylglycerol extracted from interferon-treated cells stimulates patterns of cytosolic protein phosphorylation akin to those seen in extracts of cells treated with activators of protein kinase C. Signal transduction of interferon appears to involve the breakdown of lipids to produce second messenger molecules like diacylglycerol which in turn activate protein kinase(s). Inhibitors of protein kinase C, such as H7, inhibit the antiviral action of interferon in human fibroblasts. Phospholipase C as well as calcium ionophore can produce an antiviral-like state in human fibroblasts. The data apparently suggest that these second messenger molecules are involved in the signal transduction of interferon. However, protein kinase C activators such as phorbol ester and 1-oleoyl-2-acetyl-glycerol, by themselves cannot produce an antiviral state in cells although in one report, down-regulation of protein kinase C by phorbol ester also down-regulated the interferon response. The current data suggest that diacylglycerol and protein kinase C comprise one aspect of the interferon signal transduction mechanism. More data are required to show if these rapid signalling responses of cells to interferon are responsible for its action.

F 137 Interleukin 2 (IL2) Induces Phosphorylation and Activation of p68-74 Raf Kinase in T lymphocytes, B.C. Turner, G. Hiedecker*, U. Rapp* and J.C. Reed, Path. Dept., U. of P., Phila., PA 19104 and NCI*, Frederick, MD 21701. IL2 is a critical regulator of lymphocyte proliferation. The molecular mechanisms by which IL2 transmits its signal from the membrane to the interior of the cell are poorly understood. Like most other growth factors, IL2 causes rapid phosphorylation of proteins within its target cells, however, the known subunits of the IL2 receptor lack kinase activity and no kinases have been unequivocally identified whose activities are regulated by IL2. A proto-oncogene that has been implicated in growth factor-induced signal transduction and that is expressed in T cells is c-raf, the cellular homolog of the MSV 3611 retroviral transforming gene. C-raf encodes a 68-74 kd cytoplasmic serine/threonine-specific kinase. Using an IL2-dependent murine T cell line, CTLL-2, we found that IL2 stimulated increased phosphorylation of the raf protein. Furthermore, phosphorylation of c-raf protein was accompanied by 5-10 fold elevations in raf kinase activity, as measured in raf-containing immunoprecipitates. The IL2-induced elevations in raf kinase activity occurred in a concentration-dependent manner and correlated with increased cellular proliferation. This IL2-induced activation of the raf kinase was specific, since IL4 produced no detectable increase in raf kinase phosphorylation and activity, yet did stimulate proliferation of CTLL-2 cells. Though the phorbol ester TPA was capable of inducing low levels of p68-74 raf phosphorylation and kinase activation, Protein Kinase C (PKC) depletion indicated that PKC is not necessary for IL2-induced activation of raf kinase. Taken together, these findings provide a direct link between IL2 and a kinase of known importance for regulating cellular growth.

F 138 HORMONAL REGULATION OF 1-2 DIACYLGLYCEROL CONTENT OF BOVINE ADRENAL FASCICULATA-RETICULARIS CELLS.

I.VILGRAIN, E.LIAUDET, I.GAILLARD and E.M.CHAMBAZ. INSERM 244, B.R.C.E./ L.BIO./ D.R.F./ C.E.N.G. 85 X. 38041 GRENOBLE Cedex . FRANCE

Angiotensin II (AII) but not Adrenocorticotropin hormon (ACTH) induces a break down of phosphatidylinositol 4-5, bisphosphate in adrenal fasciculata reticularis cells and activates two different messengers systems: Ca++ via inositol phosphates and protein kinase C via 1,2-Diacylglycerol (DAG). In the present study, the A II induced change in cellular DAG levels were analysed using a DAG kinase assay. In cultured bovine adrenocortical cells, AII caused a biphasic elevation of DAG content with a detectable increase in DAG levels after 30 sec. and a decrease by 2 min. The initial DAG response was followed by a second increase reaching a maximum by 40 min The maximally effective concentration of AII was 50 nM and half maximal stimulation was achieved at 0.1 nM for the initial DAG response and 0.5 nM for the second increase in DAG level. Using (14 C) Arachidonic Acid labelling of total cellular phospholipids, we show that the first peak of DAG is labelled with (14 C) arachidonic acid in a major extent as compare to the second one.The dose-response effect of AII-stimulated DAG production is correlated with the cellular cortisol production.

F 139 NOVEL G-PROTEIN ALPHA SUBUNITS AND RECEPTORS CLONED FROM MOUSE GERM CELLS. Thomas M. Wilkie, Michael P. Strathmann and Melvin I. Simon Biology Division, Caltech, Pasadena CA 91125.

Mammalian sperm exhibit functions, such as the acrosome reaction during fertilization, which require signal transduction via G-proteins. In order to investigate information processing in differentiated germ cells we developed approaches to search for specific gene products that might midiate signalling. We expected that the spermatid precursors of mature sperm might express gene products that would specify novel G-proteins. To identify candidates, clones were obtained from mouse spermatid cDNA by PCR amplification. The PCR products of 15 different G-proteins were found, including five new G-proteins which appear to be members of three new sub-families. Some of these clones were abundantly expressed in all stages of male germ cell development but none were expressed exclusively in spermatids. Any one of these G-proteins might mediate the acrosome reaction or be involved in other signalling processes critical to germ cell deveopment. Next we attempted to identify G-protein receptors expressed in mouse spermatids. We have identified nine novel receptor sequences, two of which are expressed most abundantly in spermatids. These clones are candidates for the receptor which regulates the acrosome reaction.

Tyrosine Kinase Signalling and Immune System Mechanisms

F 200 EXPRESSION OF AN ACTIVATED *lck* GENE IN TRANSGENIC MICE: ALTERED LYMPHOCYTE SIGNALLING AND ONCOGENESIS, Kristin M. Abraham, Steven D. Levin, Jamey D. Marth, Katherine A. Forbush and Roger M. Perlmutter, Howard Hughes Medical Institute and the Departments of Immunology, Medicine, and Biochemistry, University of Washington School of Medicine, Seattle, WA 98195, and the Biomedical Research Centre, University of British Columbia, Vancouver, B.C., Canada. Accumulating evidence has implicated the lymphocyte-specific protein tyrosine kinase *lck* as a signal transduction molecule in T-lymphocyte responses, including its modulation during lymphocyte activation and its physical association with the cell surface molecules CD4 and CD8. In order to assess the functional role of *lck* during T cell development and activation, we sought to overexpress both the wild type *lck* gene and an activated version containing a single codon substitution (Y→F) at position 505 in transgenic animals under the control of the proximal *lck* promoter. These transgenes (pLGY and pLGF) are expressed preferentially in the thymus, with undetectable levels of transcripts in peripheral lymphocytes. Lymphocytes from pLGY transgenic mice retain normal staining characteristics and exhibit an extremely subtle defect in Con A stimulability in the thymus only. In contrast, the transgenic pLGF animals exhibit profound immunologic abnormalities. These can be broadly grouped into two categories based on levels of expression of the transgene: low expressing lines exhibit alterations in the signalling properties of thymocytes and in peripheral T-lymphocytes. Animals expressing higher transgene levels accumulate $CD3^{-}CD8^{low}$ cells in the thymus, and develop thymic tumors prior to 7 weeks of age. Thymocytes from $pLGF^{hi}$ but not pLGY bearing animals have dramatically increased phosphotyrosine levels, and the resulting transformed thymocytes can be maintained as in vitro cell lines. Placed in the context of the known physical properties of $p56^{lck}$, these observations support the view that the *lck* gene product assists in mediating signal transduction from the T cell antigen receptor.

F 201 **PATHWAYS REGULATING PROTEIN KINASE C ACTIVATION IN PERMEABILISED HUMAN T-LYMPHOCYTES: ANALYSIS USING A NOVEL ASSAY, Denis R. Alexander, Jonathan D. Graves, Doreen A. Cantrell and Michael J.Crumpton, Imperial Cancer Research Fund, P.O.Box 123, Lincoln's Inn Fields, London, WC2A 3PX.**

We have previously utilised a pseudosubstrate peptide inhibitor in order to investigate protein kinase C (PKC) mediated events in stretolysin-O permeabilised human T-cells (Alexander, D.R.*et al.* Biochem J. 260, 893-901, 1989). In an analogous method, we have now introduced selective peptide substrates for PKC into permeabilised T-cells, and have shown that these can be used to monitor PKC activation in response to receptor ligands. The pseudosubstrate inhibitor was used to block peptide phosphorylation induced by phorbol 12,13-dibutyrate, phytohemagglutinin, GTPγS or mAb's against the CD3 antigen, so establishing that in each case phosphorylation was due to the action of PKC. An analysis of inositol phosphate (IP) production revealed a marked disparity between the concentration dependence of GTPγS induced IP production and PKC activation. For example, at 10μM GTPγS IP production was 90% of maximum, wheras the equivalent level of PKC activation was only 47%. Our data are consistent with the concept that PKC activation in T-cells is regulated by several distinct pathways. The PKC assay that we have described enables direct quantitative estimates of PKC activity in permeabilised cells in response to signal transduction via cell surface molecules. It is expected that this method will help to clarify the way in which PKC is regulated during T-cell activation.

F 202 EXPRESSION OF EGF RECEPTOR IN K562 CELL LINE BY TRANSFECTION: HIGH BASAL ACTIVITY OF RECEPTOR KINASE, Hamish Allen, Justin J. Hsuan[1], Stella Clark[1,2], Michael D. Waterfield[1] and John Haley[1,3], National Institute for Medical Research, London NW7 1AA, U.K., [1]Ludwig Institute for Cancer Research, London W1P 8BT, U.K., [2]The Walter and Eliza Hall Institute, Victoria 3050, Aus., [3]Oncogene Science, NY 11030.
The human erythroleukemic cell line K562 normally lacks both epidermal growth factor (EGF) receptors and class I histocompatibility antigens, HLA-A,B, but synthesis of HLA-A,B is inducible with interferon-gamma. We expressed the EGF receptor in K562 cells by transfection of the receptor cDNA to investigate receptor function in erythroid cells and to determine any effect of HLA-A,B expression on receptor function. The tyrosine kinase activity of the EGF receptor in the K562 transfectants was compared to that in A431 cells. The K562 receptor kinase has high basal activity in the absence of EGF. This activity may not be due to an autocrine mechanism, since we have shown that the K562 transfectants do not synthesize TGFα. The K562 receptor has one major, constitutive phosphorylation site in vivo, a threonine residue(s) that is not the site phosphorylated by protein kinase C at Thr 654. Possible mechanisms of activation of the K562 receptor kinase are being investigated, one of which may involve this threonine phosphorylation.

F 203 cAMP MEDIATED REGULATION OF c-myc IN HUMAN B-LYMPHOID CELLS, Kristin B. Andersson, Erlend B. Smeland, Steinar Funderud and Heidi K. Blomhoff, Lab. for Immunology, Institute for Cancer Research, The Norwegian Radium Hospital, Montebello, N-0310 OSLO 3, Norway.

We have studied cAMP mediated c-myc regulation in normal human B lymphocytes and in the B-precursor cell line REH.

Activation of adenylate cyclase by the diterpene forskolin results in growth inhibition (block in late G_1 and delay in G_2) in both cell systems, concurrently with reduced c-myc mRNA and protein levels. In the cell line REH this c-myc downregulation is transient and it is not yet established whether this downregulation is necessary for, or is a consequence of the observed growth arrest.

We will present results that clarify at which level c-myc downregulation occurs.

F 204 DIFFERENTIAL EFFECTS OF INTERLEUKIN-2 AND INTERLEUKIN-4 ON PROTEIN TYROSINE PHOSPHORYLATION IN FACTOR-DEPENDENT MURINE T CELLS, James A. Augustine, Janis W. Schlager and Robert T. Abraham, Department of Immunology, Mayo Clinic, Rochester, MN 55905.
The mechanism by which interleukin-2 (IL2), a requisite factor for growth of IL2-dependent T cells, transmits a mitogenic signal to the cell interior remains unclear. In this study, anti-phosphotyrosine (p-tyr) antibodies were used to immunoprecipitate p-tyr containing proteins from three IL2-dependent T cell clones bearing either a T-cytotoxic or T-helper phenotype. IL2 stimulated rapid increases in the phosphorylation of at least 6 cellular proteins in these cell lines. Phosphorylation of 97 kDa and 57 kDa proteins was a common event in all 3 clones. Subcellular localization studies revealed that most of these phosphoproteins were cytosolic in nature. In contrast, the alternative T cell growth factor, interleukin 4, induced no detectable protein tyrosine phosphorylation in these cells, indicating that tyr kinase activation was not a requisite event for mitogenic signal transmission in growth factor-stimulated T cells. We have also demonstrated that anti-p-tyr immunoprecipitates of IL2-stimulated cytotoxic T cells contained enzymatic activity similar, if not identical, to phosphatidylinositol (PI)-3'-kinase. These results suggest that phosphorylation of PI may represent a novel mechanism of second messenger generation by IL2.

F 205 STIMULATION OF EPIDERMAL GROWTH FACTOR (EGF) RECEPTOR TYROSINE KINASE ACTIVITY BY BASIC PROTEINS AND INORGANIC IONS, Paul J. Bertics and Laura Hubler, Dept. of Physiol. Chem., Univ. of Wis., Madison, WI 53706 Because of the importance of receptor-substrate interaction in EGF signal transduction, it is critical to understand the factors involved in regulating receptor activation and substrate specificity. Thus, we have examined the effects of inorganic salts, poly-amino acids and basic proteins on EGF receptor activation and tyrosine kinase activity using membranes and highly purified receptor isolated from human A431 epidermoid cells. Using the phosphorylation of an exogenous substrate, angiotensin II, ammonium sulfate (250 mM) enhanced EGF activation by ~10-fold in membranes and stimulated purified receptor, which is already EGF-saturated, by ~6-fold. The activation seen with purified receptor involves an increase in Vmax with no change in the apparent Km for angiotensin II. This effect appears to be substrate specific, since ammonium sulfate greatly inhibits the phosphorylation of another exogenous substrate, src-peptide, without affecting self-phosphorylation. Receptor activation ranging from 2-5 fold was also seen with ammonium chloride (100 mM), potassium and sodium chlorides (100 mM)/sulfates (50 mM), protamine (2-750 µg/ml), poly-arginine (40 kDa, 1-500 µg/ml) and poly-lysine (17.3 kDa, 1-500 µg/ml), whereas poly-glutamic acid (10.6 kDa, 50-500 µg/ml) had no effect. However, not all effectors showed the substrate specificity seen with ammonium sulfate, suggesting differential modes of action. In this regard, the EGF receptor carboxy-terminus may be involved in the mediation of some of these effects since a 164 amino acid C-terminally truncated receptor is insensitive to poly-arginine and protamine, but still responds to poly-lysine and ammonium sulfate. (Supported by NIH.)

F 206 FEATURES OF THE T CELL SPECIFIC CD27 ANTIGEN, *Jannie Borst[1], Rolien de Jong[2], Cees Melief[1], René van Lier[2] and Wil Loenen[1]. [1]Divisions of Immunology, The Netherlands Cancer Institute & [2]Immunobiology, Central Laboratory of the Blood Transfusion Service, Amsterdam, 1066 CX, The Netherlands.*

The function of the CD27 molecule is unknown, but various features suggest that it plays a significant role in the events following T cell activation. First, CD27 is a T cell specific antigen. Second, it is an activation antigen. Resting peripheral T cells contain a $CD27^-$ and a $CD27^+$ subset, of which only the latter is able to give help to B cells in IgM production, suggesting a difference in maturation or activation between the subsets. Upon triggering of the CD3/TCR complex, known to activate signal transduction routes involving PKC and a tyrosine kinase, CD27 expression is induced on negative cells and strongly increases on $CD27^+$ cells, as a result of transcriptional activation. In contrast, upon activation of T cells by phorbol esters, that exclusively trigger the PKC route, CD27 expression decreases. This may imply that transcriptional regulation of CD27 gene expression is an interesting model system to study the tyrosine kinase activation route. We have cloned the genomic DNA encoding the CD27 protein. DNA sequence information will give insight in the protein structure as well as in gene regulation, while subsequent expression studies should help to elucidate the biological role of CD27 and provide insight into distinct signalling pathways and temporal events in T cell activation.

F 207 **HETEROGENEITY OF PHOSPHOLIPASE C REGULATION IN T LYMPHOCYTES, Steve G. Ward and Doreen A. Cantrell, Lymphocyte Activation Laboratory, Imperial Cancer Research Fund, London, WC2A 3PX.**
In many cells, protein kinase C (PKC) activation inhibits cellular phospholipase C thereby preventing receptor-mediated phosphatidylinositol (PI) metabolism. In T lymphocytes, the T cell antigen receptor (Ti)/CD3 complex regulates PI hydrolysis and we have examined the consequences of PKC activation on Ti/CD3-mediated PI metabolism in human peripheral blood derived T lymphocytes (T lymphoblasts) and the leukaemic T cell line Jurkat. In Jurkat cells, PI metabolism following Ti/CD3 stimulation, is inhibited by PKC activation. PKC activation also inhibits calcium-induced PI metabolism in permeabilised Jurkat cells. In marked contrast, PI metabolism following Ti/CD3 stimulation in T lymphoblasts, is not inhibited by PKC activation. The different effect of PKC stimulation on PI metabolism in Jurkat cells and T lymphoblasts reveals heterogeneity of PLC regulation in T lymphocytes. Jurkat cells and T lymphoblasts express different PKC isotypes which could explain differences in PKC regulation of PI metabolism.

F 208 FLUORESCENCE RESONANCE ENERGY TRANSFER AS A MEASURE OF EGF RECEPTOR AGGREGATION IN A431 CARCINOMA CELL MEMBRANES, Kermit L. Carraway, John G. Koland and Richard A. Cerione, Department of Biochemistry, Cell and Molecular Biology, Cornell University, Ithaca, NY 14853. Fluorescence resonance energy transfer between fluorescent-labeled epidermal growth factor molecules was used as a monitor for EGF receptor-receptor interactions in isolated plasma membranes and on the surface of intact A431 human epidermoid carcinoma cells. EGF labeled at its amino terminus with fluorescein-isothiocyanate served as the fluorescence donor in these studies, while EGF similarly labeled with eosin-isothiocyanate served as the acceptor. Both of these derivatives are active in the stimulation of the endogenous receptor tyrosine kinase activity in membranes, and in the stimulation of second messenger responses in intact cells. Using isolated plasma membranes from A431 cells, we found that the labeled growth factors alone show relatively little energy transfer (~5-10% donor quenching under the conditions employed), indicative of little receptor aggregation. However, the presence of divalent cation activators of the receptor tyrosine kinase activity significantly increases the degree of energy transfer in isolated plasma membranes (as much as 40% donor quenching). The ability of these divalent cations to induce receptor-receptor interactions correlates well with their ability to stimulate the EGF receptor tyrosine kinase activity. Studies with intact A431 cells indicated that no energy transfer occurs under conditions which maintain the integrity of the cell. However, metal-stimulated energy transfer may be regained when cells are extracted at high pH. These results suggest that intact cells impart some constraint on the ability of their EGF receptors to aggregate. Moreover, since the labeled growth factors are acting to elicit second messenger responses, it may be concluded that large scale EGF receptor aggregation is not necessary for signal transduction across the plasma membrane in these cells.

F 209 IDENTIFICATION OF A NOVEL TYROSINE KINASE *eek*, Joanne Chan and Valerie M. Watt, Department of Physiology, University of Toronto, Toronto, Canada, M5S 1A8. Protein tyrosine kinases form a superfamily of structurally related enzymes, many of which mediate biological responses of target cells to extracellular signals. We have identified a novel tyrosine kinase named *eek* (*eph* and *elk*-related kinase) using low stringency screening of a rat brain cDNA library with the kinase region of the guinea pig insulin receptor-related receptor (IRR) gene as probe. Conservation of the *eek* gene in other species was indicated by the detection of fragments hybridizing to the rat *eek* DNA probe in human and mouse genomic DNA. In addition, we used the rat *eek* DNA as probe to isolate a human genomic fragment encoding part of the *eek* gene. This human *eek* DNA was used to localize the *eek* gene to human chromosome 1. Nucleotide sequence analysis revealed that the predicted *eek* protein contains all the features conserved among protein tyrosine kinases. The predicted amino acid sequence of *eek* is most similar to two putative receptor tyrosine kinases, *eph* and *elk* (60%-70% identity), and less similar to other tyrosine kinases such as *src* (46%). This suggests that *eph*, *elk* and *eek* form a new family of putative receptor tyrosine kinases possibly including other as yet unidentified members.

F 210 COMPARATIVE CHARACTERIZATION OF THE SITES OF PHOSPHORYLATION IN CLATHRIN LIGHT CHAINS PHOSPHORYLATED BY NEURONAL PP60C-SRC AND A SPLEEN TYROSINE KINASE, H.C. Cheng*, M.R. Carpenter, M.J. Mooibroek*, L.B. Smillie, and J.H. Wang*, Department of Medical Biochemistry, University of Calgary*, and Department of Biochemistry, University of Alberta , Edmonton, AB, Canada.
Purified bovine brain clathrin light chains a and b (LCa and LCb) can be phosphorylated by both a bovine spleen tyrosine kinase and neuronal pp60c-src in vitro. Phosphopeptide maps generated by clathrin light chains phosphorylated by both kinases were identical. This suggests that both the spleen tyrosine kinase and neuronal pp60c-src phosphorylated clathrin light chains at the same sites. The spleen tyrosine kinase could phosphorylate both light chains a and b to a stoichiometry of 0.2-0.5 mol. phosphate/mol. protein with Km at 30-100μM. Phosphorylation occurred chiefly on one single tyrosine in both LCa and LCb. The major site of phosphorylation in LCa was identified to be tyrosine 89 with flanking amino acid sequence YYQESNGPTDSYAAISQ. The major phosphorylation site in LCb was found to be tyrosine 87 with amino acid sequence surrounding it as VFQEANGPTDGYAAIAQ. Even though an acidic residue is located in close vicinity to the target tyrosine residue in each sequence, both phosphorylation site sequences show no significant homology to those sequences surrounding phosphotyrosine in pp60c-src and other retroviral transforming proteins.

F 211 CSF-1 STIMULATES ASSOCIATION OF ITS RECEPTOR WITH A PHOSPHATIDYL INOSITOL KINASE, G. G. Choudhury, L.-M. Wang, L. Martinez, and A. Y. Sakaguchi. Department of Cellular & Structural Biology, University of Texas Health Science Center, San Antonio 78284-7762
Colony stimulating factor 1 is a lineage specific hemopoietin required for the growth, differentiation, and survival of macrophages and their precursors. The receptor for CSF-1 is a transmembrane glycoprotein whose intrinsic tyrosine kinase is activated by ligand binding. Three deletions were introduced into the "kinase insert" region of human CSF1R cDNA and the genes expressed in NIH3T3 cells. (Deletion mutants and inclusive amino acids are: Gly684, glycine 684-leucine 750; Gly684B, glycine 684-serine 713; Lys700, lysine 700-arginine 727). Each mutant retained tyrosine kinase activity in vitro. Immune complexes of CSF1R contained a phosphatidyl inositol kinase (PIK) activity whose reaction product was suggestive of PI 3'P (identical mobility in TLC to the product obtained with polyoma virus middle T immunocomplexes). In contrast to wild type receptor, Gly684B, and Lys700, immune complexes of Gly684 did not contain PIK activity. PIK activity was stimulated by treatment of receptor-bearing cells with human recombinant CSF-1 (kindly provided by P. Ralph, Cetus). Anti-phosphotyrosine immunoprecipitates from CSF1R bearing cells contained PIK activity as well as an 85 Kd protein whose phosphorylation on tyrosine appeared to be stimulated by CSF-1. Additional mutants are being analyzed to understand the role of the kinase insert region in PI kinase association and signal transduction by CSF1R.

F 212 **BIOSYNTHESIS OF THE PROTEIN ENCODED BY THE *c-met* PROTO-ONCOGENE,** Paolo Comoglio, Silvia Giordano, Colin S. Cooper and Maria Flavia Di Renzo, Department of Biomedical Sciences & Oncology, University of Torino, C.so Massimo D'Azeglio 52, 10126 Torino, Italy
The proto-oncogene *c-met* encodes a transmembrane protein with structural features of a growth factor receptor. We have previously shown that the *c-met* protein (c-Met) is a heterodimer of two disulphide linked chains of 50 kd (α) and 145 kd (β) (Nature 339, 155-156, 1989). In this work we have studied the biosynthesis of the *c-met* product in a gastric carcinoma cell line (GTL-16) where the *c-met* gene is amplified and overexpressed. Following metabolic labelling of the cells in the presence of tunicamycin, anti-met antibodies immunoprecipitate a protein of 150 kd. In pulse-chase experiments carried out in the absence of tunicamycin, a 170 kd product appears first. Within the next few minutes, this precursor modifies its SDS migration, probably as a consequence of modification(s) of its intra-chain disulphide bonds. After 45 min of chase, this single polypeptide precursor is cleaved to form a 50 kd α subunit and a 145 kd β subunit that are joined by disulphide bonds in an αβ complex with an apparent molecular weight of 190 kd. The presence of N-linked oligosaccharides in both the precursor and the mature protein was shown by enzymatic de-glycosylation of the immunoprecipitated proteins. The half-life of the mature protein was calculated to be approximately 5 hrs. The *c-met* protein has similar structure and biosynthesis in other human cell lines.

F 213 ALTERNATIVE SPLICING PRODUCES A NOVEL FORM OF THE *FYN*-ENCODED PROTEIN TYROSINE KINASE IN HEMATOPOIETIC CELLS, Michael P. Cooke, Katherine A. Forbush, Roger M. Perlmutter, Departments of Biochemistry, Immunology, and Medical Genetics, Howard Hughes Medical Institute, University of Washington, Seattle, WA 98185.
The *fyn* proto-oncogene encodes a 59 kDa membrane-associated protein tyrosine kinase that is implicated in the control of cell growth. Here we report that there exist two distinct *fyn*-encoded transcripts which exhibit mutually exclusive expression patterns; one form is found in thymocytes, splenocytes, and some hematolymphoid cell lines, while a second form accumulates principally in brain. The thymocyte and brain forms of $p59^{fyn}$ differ in their kinase domains, and are generated by mutually exclusive alternative splicing of exon 7. Immunoprecipitation followed by *in vitro* autophosphorylation demonstrates that both forms of $p59^{fyn}$ are enzymatically active. Retroviral vectors have been used to assess the effects of brain and thymus $p59^{fyn}$ on T-cell hybridomas. These results define a novel form of the $p59^{fyn}$ protein tyrosine kinase, with an altered catalytic domain, that apparently participates in a specialized signal transduction pathway in hematopoietic cells.

F 214 **MULTI-SITE PHOSPHORYLATION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR**, Janice L. Countaway & Roger J. Davis, *Dept. of Biochemistry, University of Massachusetts Medical Center, Worcester, MA 01655*

The hypothesis that phosphorylation of the EGF receptor is mechanistically related to the modulation of EGF receptor function was tested by replacing the major sites of serine and threonine phosphorylation (Thr^{654}, Thr^{669}, Ser^{1046}, and Ser^{1047}) with alanine residues by site-directed mutagenesis of the human EGF receptor cDNA. EGF receptors containing single point mutations or multiple mutations were expressed in Chinese hamster ovary (CHO) cells. Phorbol ester caused an inhibition of the tyrosine phosphorylation of wild- type EGF receptors and receptors lacking Thr^{669}, Ser^{1046}, or Ser^{1047}. In contrast, the inhibition of EGF receptor tyrosine phosphorylation caused by phorbol ester was not observed for any of the mutated EGF receptors that lacked Thr^{654}. These results support the hypothesis that Thr^{654} plays a central role in the control of the EGF receptor tyrosine protein kinase activity. Analysis of the role of serine and threonine phosphorylation in receptor transmodulation revealed that treatment with phorbol ester caused an inhibition of the high affinity binding of ^{125}I-EGF to cells expressing wild- type EGF receptors and each of the mutated EGF receptors examined. These data indicate that the regulation of the apparent affinity of the EGF receptor in CHO cells is independent of the major sites of phosphorylation of the EGF receptor (Thr^{654}, Thr^{669}, Ser^{1046}, and Ser^{1047}).

F 215 **$gp185^{erbB-2}$ is a constitutively active kinase which does not require dimerization to exert its transforming action.** P.P. Di Fiore, O. Segatto, I.M. Borrello, J.H. Pierce, F. Lonardo and S.A. Aaronson. LCMB, NCI, Bethesda, MD

The *erb*B-2 gene product, $gp185^{erbB-2}$, displays a potent transforming effect and constitutive tyrosine kinase activity when overexpressed in NIH/3T3 cells. This effect is unlikely to depend on the autocrine secretion of an *erb*B-2 ligand, since a chimeric molecule engineered between the *erb*B-2 extracellular domain and the intracellular domain of the EGFR was found to be biologically and catalytically inactive. Substitution of the carboxy-terminal domain of the EGFR with the analogous region of $gp185^{erbB-2}$ induced EGF independent transforming activity as well as constitutive *in vitro* and *in vivo* autophosphorylation. Conversely, the reciprocal chimera showed reduced biologic and catalytic activity when compared to the w.t. *erb*B-2 product. Thus, the upregulation of $gp185^{erbB-2}$ enzymatic activity exerted by its COOH domain can explain, at least in part, its constitutive kinase activity. At variance with other receptors, $gp185^{erbB-2}$ signalling does not depend on the formation of dimeric molecules. In fact, the normal *erb*B-2 product as well as two *erb*B-2 mutants possessing increased kinase activity are present in a monomeric state in intact cells, even when expressed 100 fold over normal levels.

F 216 LIGAND-INDUCED PHOSPHORYLATION OF THE COLONY-STIMULATING FACTOR 1 RECEPTOR OCCURS THROUGH AN INTERMOLECULAR REACTION THAT TRIGGERS RECEPTOR DOWNMODULATION, James R. Downing,[1,2] Masahiro Ohtsuka,[1] Martine F. Roussel,[1] and Charles J. Sherr,[1,3] Departments of Tumor Cell Biology[1] and Pathology[2] and Howard Hughes Medical Institute,[3] St. Jude Children's Research Hospital, Memphis, TN 38105

Ligand-induced tyrosine phosphorylation of the human colony-stimulating factor 1 receptor (CSF-1R) could involve either an intra- or intermolecular mechanism. To distinguish between these possibilities, we examined the ability of a CSF-1R carboxylterminal truncation mutant to phosphorylate a kinase-defective receptor, CSF-1R[met 616], that contains a methionine-for-lysine substitution at its ATP binding site. Using an antipeptide serum that specifically reacts with epitopes deleted from the enzymatically competent truncation mutant, cross-phosphorylation of CSF-1R[met 616] on tyrosine was demonstrated, both in immune complex kinase reactions and in intact cells stimulated with CSF-1. Both in vitro and in vivo, CSF-1R[met 616] was phosphorylated at sites identical to those in wild-type CSF-1R, suggesting that receptor phosphorylation on tyrosine normally proceeds via an intermolecular interaction between receptor monomers. When expressed alone, CSF-1R[met616] did not undergo ligand-induced downmodulation, but its phosphorylation in cells coexpressing the kinase-active truncation mutant accelerated its degradation. These results suggest that binding of CSF-1 induces receptor aggregation, leading to the activation of the receptor kinase, intermolecular phosphorylation of receptor subunits, and downmodulation.

F 217 A NOVEL CYTOPLASMIC TYROSINE KINASE GENE, blk, IS SPECIFICALLY EXPRESSED IN B-LYMPHOID CELLS, Susan M. Dymecki+,#, John E. Niederhuber*,+ and Stephen V. Desiderio+,#, +Department of Molecular Biology and Genetics, #Howard Hughes Medical Institute Laboratory of Genetics and *Departments of Surgery and Oncology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205.
Several pathways of transmembrane signaling in lymphocytes are known to involve protein-tyrosine phosphorylation. With the exception of $p56^{lck}$, a T-lymphoid tyrosine kinase of the Src family that associates with the T-cell transmembrane proteins CD4 and CD8, the kinases that function in these pathways are unknown. By screening a mixed B- and T-cell cDNA library with degenerate oligonucleotides derived from nucleotide sequences conserved among four cytoplasmic tyrosine kinase genes (lck, v-abl, c-src and v-yes), we isolated a murine lymphocyte cDNA that represents a novel B-lymphoid-specific member of the src family . This cDNA, which we call blk (for B-lymphoid kinase), specifies a 55,000 MW protein-tyrosine kinase that is related to but distinct from previously identified products of retroviral or cellular genes. Overall, the protein encoded by blk ($p55^{blk}$) exhibits 61% and 63% amino acid sequence identity to the products of lck and hck, respectively. In the mouse and among immortalized cell lines, blk is specifically expressed in the B-cell lineage. The B-lymphoid specificity of blk expression distinguishes it from other members of the src family. We propose that $p55^{blk}$ is a B-lymphoid-specific signal transduction element analogous to the T-cell tyrosine kinase $p56^{kk}$.

F 218 EVIDENCE FOR THE INVOLVEMENT OF A CHOLERA-TOXIN-SENSITIVE G-PROTEIN IN V-SRC- AND V-FPS-INITIATED SIGNAL TRANSDUCTION: David A. Foster, Konstantina Alexandropoulos, Sajjad A. Qureshi, and Rudolph Spangler. The Institute for Biomolecular Structure and Function and The Department of Biological Sciences, The Hunter College of the City University of New York, 695 Park Avenue, New York, New York 10021.

V-Src and v-Fps induce expression of the transformation-related 9E3 gene in chicken embryo fibroblasts with a sensitivity to protein kinase inhibitors consistent with a protein kinase C (PKC) mediated pathway. GTPγS and cholera toxin also induce 9E3 gene expression. G_s is known to be activated by cholera toxin and transduces signals by activating adenylyl cyclase - leading to the production of cAMP and the activation of protein kinase A (PKA). However, induction of 9E3 by cholera toxin appears to be independent of cAMP and PKA. To test if a G-protein were part of a v-Src and/or v-Fps induced signal transduction pathway, we tested the sensitivity to the G-protein inhibitor GDPβS. GDPβS inhibited both v-Src- and v-Fps-induced 9E3 gene expression. In contrast, GDPβS did not inhibit TPA induced 9E3 gene expression. These data suggest that a putative G-protein is functioning either upstream from or independent of PKC in v-Src and v-Fps mediated induction of 9E3 gene expression.

F 219 A 180000 DALTON GLYCOPROTEIN IS AN INSULIN RECEPTOR TYROSINE KINASE SUBSTRATE, H. Joseph Goren, Donna Boland, Department of Medical Biochemistry, University of Calgary, Calgary, AB T2N 4N1, Canada.
Several laboratories have demonstrated that proteins in the 150-195 kiloDalton (kDa) range are substrates for the insulin receptor (IR) tyrosine kinase. We have studied protein phosphorylation in wheat germ agglutinin (WGA) purified plasma membranes from several IR-containing tissues and with the exception of rat adipocytes have found a 180 kDa phosphorylated protein (pp180). Human placenta, and rat liver, muscle, heart, brain and adipocyte membrane proteins were phosphorylated 10 min, 20 C in the presence of 50 uM ATP, 5 mM $MnCl_2$ and in the presence or absence of 10 nM insulin. The reaction was terminated with 0.2 mM vanadate and 5 mM EDTA. Rabbit antiphosphotyrosine antisera-immunoprecipitated proteins were seperated by dodecyl sulphate gel electrophoresis and detected autoradiographically. In addition to phospho-IR β-subunit, pp180 was detected. In the absence of reducing agents, pp180 migrated as a 150 kDa protein. The addition of neuraminidase to pp180 decreased its size to 170 kDa. Reverse-phase high pressure liquid chromatography of trypsin-digested placenta and rat liver pp180 yielded identical chromatograms of 10 phosphopeptides. Phosphotyrosine along with phosphoserine in some and phosphothreonine in other tryptic-peptides were found. Insulin increased phosphate incorporation into all peptides but mostly into 5 tryptic-peptides. 9 of 10 tryptic peptides were susceptible to staphylococcal aureus V8 protease. Preimmunoprecipitation of placenta IR with an anti-IR monoclonal antibody prevented completely protein phosphorylation. Maximal and 50% insulin-stimulated phosphorylation of β-subunit and pp180 occurred at ~10 and ~3 nM, respectively. Glycerol gradient centrifugation of tissue homogenates, demonstrated IR and pp180 in endosomal and plasma membrane fractions. These results suggest that many insulin receptor containing tissues contain a 180 kDa glycoprotein: (i) that is a single polypeptide with intramolecular disulfide bonds, (ii) that is associated with endosomal and plasma membranes, (iii) that has several tyrosine kinase sensitive sites, and (iv) that is a substrate for the insulin receptor tyrosine kinase. Some evidence suggests that pp180 may be similar to hepatoma cytoplasmic pp185, an IR substrate which functions in insulin signal transduction (Cell 54: 641, 1988).

F 220 T CELL ANTIGEN RECEPTOR COUPLING TO INOSITOL PHOSPHOLIPID HYDROLYSIS AND REGULATION OF PROTEIN KINASE C IN PERMEABILIZED HUMAN T LYMPHOCYTES.

J.D.Graves and D.A.Cantrell. Lymphocyte Activation Laboratory, Imperial Cancer Research Fund, P.O. Box 123, Lincoln's Inn Fields, London, WC2A 3PX, U.K.

Triggering of the T cell antigen receptor complex (Ti/CD3) activates a phospholipase C inducing the breakdown of inositol phospholipids. In streptolysin O permeabilized T lymphocytes, inositol phosphate production can be stimulated in response to the guanine nucleotide analogue GTPγS and the anti-receptor antibody UCHT1, allowing investigation of the role of G proteins in coupling Ti/CD3 to PLC. GDPβS, a guanine nucleotide analogue which antagonises G protein mediated events, inhibited both UCHT1 and GTPγS-induced inositol phospholipid hydrolysis, implicating a G protein in Ti/CD3 coupling. The effects of GTPγS and UCHT1 on inositol phospholipid metabolism were neither additive nor synergistic, suggesting that stimulation of the Ti/CD3 complex does not induce exchange of exogenous guanine nucleotides. In permeabilized cells, PKC mediated phosphorylation can be induced by receptor stimulation with PHA, the phorbol ester PDBu and GTPγS. PHA induced inositol phospholipid hydrolysis correlated closely with PHA induced phosphorylation, suggesting that PHA regulates PKC activation via diacylglycerol produced as a consequence of inositol phospholipid hydrolysis. In contrast, there was a discrepancy between GTPγS effects on phosphatidylinositol turnover and PKC activation. The half maximal GTPγS concentration for inositol phosphate production and PKC activation was 0.75μM and 75μM respectively. The data are consistent with the idea that other G protein-regulated signal transduction pathways, in addition to those involving inositol phosphate production, exist for the regulation of PKC in T lymphocytes.

F 221 INHIBITION OF PDGF-INDUCED CELL SIGNALS AND DNA SYNTHESIS BY THE TYROSINE KINASE ANTAGONIST GENISTEIN. Timothy D. **Hill**, Lawrence J. **Mordan** and Alton L. **Boynton**. Cancer Research Center of Hawaii, Basic Science, University of Hawaii, Honolulu, HI, 96813.

To determine what role tyrosine kinase plays in growth factor mediated cell signaling and in the progression of a cell through its growth-division cycle, we treated cultured C3H-10T1/2 mouse fibroblasts with the selective tyrosine kinase blocker genistein and then monitored changes in calcium levels, protein phosphorylation and DNA synthesis stimulated with PDGF (c-sis). Addition of 5 ng/ml of PDGF to fura-2 loaded cells stimulated a 6-fold rise in the calcium concentration, which was blocked by genistein (IC50 = 30 uM). Similarly, the rapid phosphorylation of an 80 kDa protein substrate induced by PDGF was inhibited by genistein. We exploited the reversible nature of genistein inhibition in DNA synthesis experiments. Control and genistein-treated (100 uM) cells stimulated with 10 ng/ml PDGF were transferred to fresh medium at various time points and allowed to proceed in the presence of [3H]thymidine for the remainder of the 24 hour period. Once washed free of genistein the cells progressed through their growth-division cycle, although no change in the calcium levels or protein phosphorylation was observed after removal of genistein. These DNA synthesis studies also revealed two periods during the G1 phase of the cell cycle that require tyrosine kinase. The first period (early G1) occurs during the 4 hours immediately following stimulation by PDGF, and the second period (late G1) occurs 2 to 3 hours prior to DNA synthesis. These results suggest that although early cell signals are generated by a mechanism coupled to growth factor receptor tyrosine kinase, progession through the cell cycle only requires activation of tyrosine kinase.

F 222 ALTERATIONS OF THE LYMPHOCYTE-SPECIFIC PROTEIN TYROSINE KINASE, $p56^{lck}$, IN RESPONSE TO IL-2, I.D. Horak[1,2], P.J. Lucas[3], E.M. Horak[1], R.E. Gress[3], and J.B. Bolen[1]. Laboratory of Tumor Virus Biology[1], Medicine Branch[2], and Experimental Immunology Branch[3], National Cancer Institute, Bethesda, Maryland 20892.

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

F 223 THE *trk*B TYROSINE PROTEIN KINASE LOCUS CODES FOR TWO CLASSES OF CELL SURFACE RECEPTORS, ONE OF WHICH LACKS THE KINASE CATALYTIC DOMAIN: DIFFERENTIAL EXPRESSION IN NEURAL TISSUES. Rüdiger Klein[1], Luis Parada[2] and Mariano Barbacid[1], [1]Department of Molecular Biology, Squibb Institute for Medical Research, Princeton, NJ 08543, [2]Molecular Embryology Section, BRI, NCI-FCRF, Frederick, MD 21701
Screening of a mouse brain cDNA library with a probe containing sequences from the tyrosine kinase domain of the human *trk* proto-oncogene has led to the isolation of a highly related gene, designated *trk*B (EMBO J., Dec. 89). *In situ* hybridization studies have indicated that *trk*B is expressed in the central (CNS) and peripheral (PNS) nervous systems of both fetal and adult mice. However, the *trk*B locus exhibits a complex pattern of transcription. At least six RNA species ranging in size from 9 kb to 2 kb have been identified in brain. Molecular analysis of cDNA clones derived from these *trk*B transcripts revealed that only two RNA species (9 kb and 5.5 kb) code for a cell surface receptor containing a tyrosine protein kinase catalytic domain. Expression of cDNA clones derived from these transcripts revealed a membrane-associated glycoprotein of 145 kd which displays the expected tyrosine protein kinase activity. In contrast, the other four transcripts code for glycoproteins of 95 kd that contain the *trk*B ligand-binding domain anchored to the cellular membrane, but lack a catalytic cytoplasmic domain. Using probes specific for each RNA species, we have shown that these two classes of cell surface receptors are differentially expressed in adult mouse brain. For instance, the pyramidal cell layer of the hippocampus exclusively contains those transcripts encoding the kinase catalytic domain. In contrast, structures corresponding to the choroid plexus of the 3rd and lateral ventricles only hybridize to probes derived from those mRNAs coding for the short ligand-binding form of the *trk*B receptor. These findings suggest an important role of the *trk*B locus in neurogenesis.

F 224 *PERTUSSIS* TOXIN AND ITS B OLIGOMER STIMULATE MITOGENESIS AND CALCIUM INFLUX IN HUMAN B LYMPHOCYTES, Jean-Pierre Kolb*, Elisabeth Génot*, Elisabeth Petit-Koskas*, Nathalie Paul-Eugène° and Bernard Dugas°. * U196 INSERM, Institut Curie, Paris and ° Institut Beaufour, Les Ulis, France.

Pertussis toxin was found to stimulate DNA synthesis in human B lymphocytes, either quiescent or activated by a first signal mimicking the antigen, such as anti-μ antibody, or the SAC mitogen. This mitogenic effect was observed at concentrations above 100 ng/ml and reached a plateau at 1000 ng/ml. The peak of stimulation was obtained 3-4 days after the initiation of the culture. The toxin alone was unable to trigger a true multiplication of the B cells but elicited an increased survival. Similar effects to those evoked by the whole intact toxin were obtained in the presence of its binding or "B" protomer. By contrast, the "A" or catalytic unit, which induces in B lymphocytes the ADP- ribosylation of a Gi-like protein, was devoid of mitogenic activity.

Both the intact toxin and its "B" subunit, but not the "A" oligomer, were able to trigger an increase in the intracytoplasmic concentration of calcium in B cells. This increase in $(Ca^{++})_i$ was exclusively due to calcium influx and not to the release from intracellular stores.

Besides, stimulation of DNA synthesis was not modified by addition of staurosporine, an inhibitor of the protein kinase C, while preincubation with the calcium/calmodulin complex antagonist W7 resulted in a marked impairment of the proliferative response.

A possible role for the soluble cleaved form of the CD23 antigen (RFcεII) in the process of *Pertussis* toxin-induced mitogenicity will be presented. Our results may contribute to explain the long-known adjuvant effect of *Pertussis* toxin in antibody production.

F 225 THE ROLE OF A TYROSINE PHOSPHATASE, CD45, IN PROXIMAL SIGNALLING VIA THE T CELL ANTIGEN RECEPTOR, G. Koretzky, J. Picus, and A. Weiss, Department of Microbiology and Immunology, and HHMI University of California, San Francisco, San Francisco, CA 94143. Stimulation of T lymphocytes via their antigen receptor (TCR) results in the activation of a tyrosine kinase and the generation of inositol phosphate (IP) derived second messengers. The relative importance of these pathways in initiating distal biologic events and how they interact remains unclear. We have isolated a variant of the human T cell leukemic line, HPB-ALL, which lacks reactivity with a panel of monoclonal antibodies directed against CD45, a cell surface protein tyrosine phosphatase. Furthermore, membrane preparations from this clone show markedly reduced tyrosine phosphatase activity when compared to CD45 positive cells. Unlike variants expressing CD45, stimulation of the TCR in this cell does not result in IP generation nor in more distal markers of cellular activation. Interestingly, a component of the TCR itself, the zeta chain, is constitutively phosphorylated on tyrosine residues in the non-signalling, CD45 negative variant. These data suggest that a tyrosine phosphatase, perhaps through the regulation of zeta chain phosphorylation, may play a crucial role in the ability of the TCR to generate IP.

F 226 ISOLATION AND CHARACTERIZATION OF *erb*B-3 DEFINE ITS PREDICTED PROTEIN AS A NEW MEMBER OF THE *erb*B/EGF RECEPTOR TYROSINE KINASE SUBFAMILY, Matthias H. Kraus, Lorella Marazzi, Armando DiDonato, Wolfgang Issing, Toru Miki, Nicholas C. Popescu, and Stuart A. Aaronson, National Cancer Institute, Bethesda, MD 20892.
Proto-oncogenes encoding growth factor receptors constitute distinct families with close overall structural homology. Based upon accumulating evidence implicating EGF-R and *erb*B-2 in normal growth regulation as well as the neoplastic process, we searched for additional members in this receptor tyrosine kinase subfamily. A related DNA fragment distinct from the EGF-R and *erb*B-2 genes was detected by reduced stringency hybridization of v-*erb*B to normal genomic human DNA. Characterization of three genomic exons determined their closest homology of 64% and 67% to a contiguous region within the tyrosine kinase domains of the EGF-R and *erb*B-2 proteins, respectively. cDNA cloning revealed a predicted 148 kd transmembrane polypeptide with structural features identifying it as a novel receptor-like kinase of the *erb*B family, prompting us to designate the new gene as *erb*B-3. It was mapped to human chromosome 12q13 and was shown to be expressed as a 6.2 kb transcript in a variety of normal tissues of epithelial origin. Markedly elevated *erb*B-3 mRNA levels were demonstrated in certain human mammary tumor cell lines suggesting that increased *erb*B-3 expression may play a role in some human malignancies. Studies investigating the biological function of *erb*B-3 are in progress.

F 227 MECHANISM OF LIGAND ACTIVATION IN THE CSF-1 RECEPTOR. Angel W. Lee and Arthur W. Nienhuis, Clinical Hematology Branch, NIH, Bethesda Md. 20892. The macrophage colony stimulating factor (CSF-1) acts on its receptor, a 160 kD surface glycoprotein encoded by the protooncogene c-fms, to activate it as a tyrosine kinase. Two models have been proposed for the mechanism of kinase activation: an intra versus an intermolecular model. They differ in that in the former, conformational changes induced by ligand binding in the external domain must be transmitted to the transmembrane region, whereas in the latter, the role of such conformational changes might be to stabilize receptor-receptor interactions. To discriminate between the two, mutants have been constructed in which insertions have been introduced at the junction between the external and transmembrane domains. The first consisted of 7 (Glu-Ala) units predicted to assume a rigid alpha helix. The second consisted of 12 glycines predicted to adopt a flexible configuration. Both insertions were designed to structurally isolate the external domain, while allowing for receptor oligomerization. Retroviral vectors were introduced into 32D cells, an IL-3 dependent myeloid cell line. 125I-CSF-1 binding showed that the mutant receptors were expressed on the cell surface, and had an affinity similar to wild type. In vivo 32P labelling followed by immunoprecipitation with an antiphosphotyrosine antibody demonstrated that the mutants exhibited ligand-stimulated autophosphorylation which by phosphoaminoacid analysis was shown to occur on tyrosines. A potential intracellular substrate for the CSF-1 receptor was also identified. 3H-thymidine uptake experiments showed that CSF-1 stimulated DNA synthesis in wild type and mutant cells. Finally, cells expressing wild type or mutant receptors were able to proliferate in a CSF-1-dependent fashion in methyl-cellulose. Thus despite large insertions at the junction between the ligand binding domain and trans-membrane region, an area that must be critical in an intramolecular activation model, the mutants function normally. Our data are therefore most consistent with an intermolecular mechanism of ligand activation.

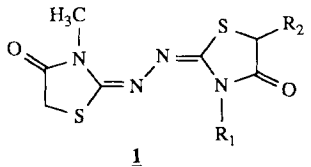
F 228 IL 3 AND GM-CSF ACTIVATION OF SERINE AND TYROSINE KINASES IN HUMAN AND MURINE MYELOID CELLS, Diana Linnekin and William L. Farrar, Laboratory of Molecular Immunoregulation, BRMP, DCT, NCI-FCRF, Frederick, Maryland 21701-1013, USA. Cytokine induced protein kinase activation was examined in the cell lines AML 193 (human origin) and NSF60.8 (murine origin). Cells radiolabeled with 32P-orthophosphate were stimulated with GM-CSF or IL 3 and lysed after the appropriate stimulation period. Assessment of tyrosine kinase activation was performed by immunoprecipitating cell lysates with monoclonal antibody to phosphotyrosine, while analysis of serine phosphorylation was performed on whole cell lysates. Phosphoproteins were resolved using 2 dimensional gel electrophoresis. A 68 kDa protein (pI 5.0) was phosphorylated on serine residues in both AML-193 and NSF60.8 cells after stimulation with GM-CSF or IL-3. In addition, treatment with phorbol myristate acetate (PMA) resulted in phosphorylation of the identical protein in both the murine and human cells. Stimulation of both AML-193 and NSF60.8 cells with IL 3 or GM-CSF resulted in phosphorylation of a protein of approximately 130-150 kDa on tyrosine residues. Kinetics of this response were rapid, increases were noted within 30 seconds of cytokine stimulation. PMA treatment did not stimulate phosphorylation of p140. The identification of p68 as a common substrate for a kinase activated by both PMA and the cytokines GM-CSF/IL 3 suggests a possible role for PKC in the signal transduction of these molecules. Depletion of PKC isozymes through pretreatment with high concentrations of PMA indicate PKC is not necessary for the tyrosine kinase activation but may be involved in phosphorylation of the p68 serine substrate. These data indicate that activation of serine and tyrosine kinases by IL 3 and GM-CSF are coordinate yet independent events.

F 229 PURIFICATION AND COMPARATIVE CHARACTERIZATION OF A BOVINE SPLEEN TYROSINE KINASE AND BOVINE NEURONAL pp60src, Craig M.E. Litwin, H.C. Cheng and J.H. Wang, Department of Medical Biochemistry, University of Calgary, Calgary, Alberta, Canada, T2N 4N1.
Tyrosine kinase activity has been implicated in the regulation of cellular proliferation and differentiation. We have purified tyrosine kinase activity from bovine brain and spleen. The purification involves multiple chromatographic steps and a key feature of the work-up is the use of an anti-phosphotyrosine affinity column. The resulting preparation is of relatively high purity; the spleen prep showed a tight cluster of bands at 55-60 KDa while the brain kinase prep showed a somewhat similar pattern. The kinases each behave differently during the purification suggesting they are distinct from each other. A comparision of various features of the two kinase was undertaken. A 61 KDa species from bovine brain, migrating slightly above pp60 src from chicken embryo fibroblasts, was immunoprecipitated with an anti-src antibody (Mab 327) however, the antibody did not interact with the spleen kinase. This suggests that the brain kinase is pp60 src. Comparative peptide mapping of the spleen and brain kinase autophosphorylation site was performed to assess the interrelatedness of the two kinases. The ability of the two kinases to undergo a reversible autophosphorylation reaction was also investigated.

F 230 EXTRACELLULAR CONTROL OF ERYTHROCYTE METABOLISM MEDIATED BY A CYTOPLASMIC TYROSINE KINASE. Philip S. Low[1], Robert L. Geahlen[2], Ernest Mehler[3], and Marietta L. Harrison[2]. Departments of Chemistry[1] and Medicinal Chemistry and Pharmacognosy[2], Purdue University, W. Lafayette, IN 47907 USA, and Biozentrum der Universität Basel, CH-4056 Basel, Switzerland[3].
We wish to propose a new mechanism of metabolic regulation mediated by a cytoplasmic tyrosine kinase. Briefly, as Steck et al. have shown, we propose that glyceraldehyde-3-phosphate dehydrogenase (G3PDH) associates reversibly with the N-terminus of the cytoplasmic domain of band 3. Once the enzyme is bound, it is totally inhibited; however, upon release it is restored to full activity. We demonstrate that control of enzyme binding and consequently the glycolytic flux through this control point is executed by phosphorylation of Tyr 8 and Tyr 21 within the glycolytic enzyme binding site on band 3. This phosphorylation results in obstruction of enzyme binding, leading to G3PDH activation. Although not essential to the hypothesis, molecular modeling studies reveal that G3PDH interacts with band 3 like a "donut on a string" in a manner that is sterically prohibited by phosphorylation of band 3. The tyrosine kinase involved in band 3 phosphorylation is further demonstrated to be regulated by receptors located in the plasma membrane of the erythrocyte. Any agent which activates the tyrosine kinase is shown to coordinately activate red cell glycolysis. Conversely, any pharmaceutical which blocks tyrosine phosphorylation of band 3 also blocks stimulation of glucose metabolism. The change in profile of glycolytic intermediates resulting from stimulation of the kinase reveals a cross-over at the G3PDH reaction, confirming G3PDH as the site of this regulation. Thus, while steady state red cell metabolism may be regulated by conventional feedback inhibition, external modulation of the glycolytic flux is likely controlled by tyrosine kinase regulation of the inhibitory association of G3PDH with band 3.

F 231 THIAZOLIDINE-DIONES - A NOVEL CLASS OF SELECTIVE TYROSINE KINASE INHIBITORS, Lydon N., Geissler J., Roesel J., Regenass U., Meyer Th., Storni A. and Traxler P., Research Department, Pharmaceuticals Division, CIBA-GEIGY Ltd., Basle, Switzerland.

A series of thiazolidine-dione derivatives of general formula **1** have been synthesized and tested against a pannel of protein kinases. The derivatives CGP 520 (R_2: $=CH_2$), CGP 645 (R_2: $=CH_2$) and CGP 4303 (R_2: $-CH_2N(CH_3)_2$ have been found to selectively inhibit the EGF-R tyrosine kinase (IC_{50}: 1.3 - 5.7 µM) relative to other tyrosine and serine/threonine protein kinases. In a cellular test system using an EGF-dependent mouse epidermal keratinocyte cell-line (MK-line), CGP 520, CGP 645 and CGP 4303 showed potent anti-proliferative effects (IC_{50}: 1 - 4.2 µM). Structure-activity relationships within this class of compounds and mechanistic studies with respect to enzymatic and cellular activity will be discussed.



1

	R_1	R_2
CGP 645	$CH_2CH=CH_2$	$=CH_2$
CGP 520	$CH_2C(CH_3)=CH_2$	$=CH_2$
CGP 4303	$CH_2C(CH_3)=CH_2$	$CH_2-N(CH_3)_2$

F 232 LIVER-SPECIFIC PROTEIN-TYROSINE KINASE, *tec*, IS OVEREXPRESSED IN HEPATOCELLULAR CARCINOMA, Hiroyuki Mano*, Fuyuki Ishikawa, Junji Nishida, Hisamaru Hirai and Fumimaro Takaku, The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. * Present address; Department of Biochemistry, St Jude Children's Research Hospital, 332 North Lauderdale P.O.Box 318, Memphis TN 38101-0318 USA. We have isolated a novel murine cDNA, *tec* (*t*yrosine kinase *e*xpressed in hepatocellular *c*arcinoma). Nucleotide sequence of this cDNA shows that c-terminal domain of its predicted protein has significant homology with the members of the *src* family. This protein does not contain the transmembrane portion. It has SH 2 and SH 3 regions, though the homology of these sites with the *src* family is relatively low. Genomic analysis reveals that the *tec* gene might be conserved among human, mouse, chicken and *Xenopus laevis*. This gene is mainly expressed in liver, and faintly in ovary and kidney. Northern analysis further shows that in several cell lines of human hepatocellular carcinoma the *tec* gene is overexpressed compared to normal human liver. This gene might play a key role in the cell growth of hepatocytes or in hepatocarcinogenesis. Although this gene is little expressed in bone marrow or spleen of normal mouse, the *tec* gene is, interestingly, highly expressed in several hematopoietic cell lines of mouse. The role of the *tec* gene in leukemogenesis is under investigation.

F 233 ANALYSIS OF POTENTIAL EFFECTOR PROTEINS INVOLVED IN V-ERB B MEDIATED TRANSFORMATION. Deborah J. McCarley and Randall C. Schatzman, Institute of Cancer and Developmental Biology, Syntex Research, Palo Alto, CA 94304. The role of tyrosine-specific phosphorylation in v-erb B mediated transformation was examined utilizing a polyclonal antibody specific for phosphotyrosine together with immunoprecipitation and immunoblotting techniques. The major phosphotyrosine containing proteins detected in v-erb B transformed cells were $gp68^{v\text{-}erb}$ and $gp74^{v\text{-}erb}$. In addition, the phosphotyrosine antibodies detected a number of cellular proteins that showed either enhanced phosphorylation or were newly phosphorylated on tyrosine residues in response to v-erb B mediated transformation. These proteins migrated at apparent molecular weights of 170, 145, 90, 85, 75, 65, 52, 42, 37, and 30 Kd. In order to further assess how the phosphotyrosine content of these cellular proteins correlated with transformation, we used the phosphotyrosine antibodies to compare proteins in cells expressing various mutants of v-erb B. The mutant proteins examined included a soluble, non-transforming form of v-erb, a v-erb B protein with an inactive ATP binding domain, two proteins with substitutions at potential sites of tyrosine phophorylation, and a v-src/v-erb chimera. The results demonstrated that phosphorylation of some cellular proteins appeared to correlate with the ability of v-erb B to transform cells. Comparison of v-erb B and v-src transformed cells showed that there appeared to be phosphotyrosine containing proteins that are in common and those which are unique to cells transformed by either of these oncogenes. We are currently attempting to identify those proteins whose change in phosphotyrosine content correlates with transformation; among these proteins, our evidence suggests that the EGF receptor, PLC-δ, and c-raf all demonstrate enhanced phosphorylation in response to v-erb B transformation.

F 234 INTERLEUKIN 2 RECEPTOR ASSOCIATED TYROSINE KINASE, Isabel Merida and Glen N. Gaulton. Department of Pathology and Laboratory Medicine, Division of Immunobiology. University of Pennsylvania, School of Medicine, Philadelphia PA 19104. The addition of interleukin 2 (IL2) to the IL2-dependent murine cytotoxic T cell line CTTL-2 induced increased tyrosine phosphorylation of a protein with a molecular weight of 80,000 and, to a lesser extent, proteins with molecular weights of 130,000, 100,000 and 69,000. To correlate the stimulation of tyrosine phosphorylation with increased tyrosine kinase activity, cell free phosphorylation assays were performed. Phosphotyrosine containing proteins were purified from detergent solubilized cell lysates by immunoprecipitation with anti-phosphotyrosine antibodies. IL2 treatment of cells increased H2B phosphorylation 10-fold when compared to non stimulated cells. Phosphorylation was first detected after 2.5 min of incubation with physiologically relevant (100 pM) IL2 doses. To examine if tyrosine kinase activity was resident within the IL2 receptor complex, cell-free phosphorylation assays were performed with ligand-receptor complexes following cross-linking with IL2 and purification by immunoabsorption with an anti-IL2 antibody. Tyrosine kinase activity was found specifically associated with the IL2 receptor complex. These results indicate that the IL2 receptor complex contains a tyrosine kinase activity that is induced by IL2 binding and suggest that components of the complex may be a substrate of this activity.

F 235 TYROSINE SPECIFIC PROTEIN PHOSPHORYLATION IN POLYMORPHONUCLEAR LEUKOCYTE (PMN) ACTIVATION, F.H. Mermelstein, D.L. Laskin, A.A. Sirak and J.D. Laskin, Environmental & Community Medicine, UMDNJ-RWJ Med. School and Pharmacology &Toxicology, Rutgers University., Piscataway, NJ 08854.
We are using a highly specific monoclonal antibody (Mab), DL1.2, to characterize biochemical mechanisms underlying PMN activation. This Mab interacts with a unique glycoprotein (MW=120 kD) present on mature PMN and induces chemotaxis and oxidative metabolism. We found that fMLP, but not DL1.2 induces a rapid and transient mobilization of intracellular Ca^{++} in PMN. In addition, chemotaxis induced by fMLP but not DL1.2 is blocked by pretreatment of the cells with pertussis toxin. Chemotaxis in response to DL1.2 is partially blocked by protein kinase C (PKC) inhibitors, but unaffected by preincubation of the cells with phorbol myristate acetate, which activates PKC. This suggested that other protein kinases are involved in PMN chemotaxis induced by DL1.2. Using a Mab to phosphotyrosine (pTyr) in proteins, we analyzed Tyr specific protein phosphorylation in PMN. Flow cytometry revealed that the anti-pTyr antibody bound to a homogeneous population of unstimulated PMN. Activation of PMN by DL1.2, but not fMLP, resulted in the appearance of a second population of pTyr positive cells. Immunoprecipitates of ^{32}P-labeled PMN demonstrated that DL1.2, but not fMLP stimulates phosphorylation of two high MW (100-150 kD) pTyr containing proteins. These data suggest that Ca^{++} mobilization is not required for chemotactic factor induced signal transduction and that Tyr phosphorylation may be important in PMN activation induced by DL1.2.

F 236 THE ROLE OF ARACHIDONIC ACID RELEASE IN T CELL RECEPTOR-MEDIATED SIGNALLING OF CYTOTOXIC T CELLS, Anne M. O'Rourke, Joy D. Rogers and Matthew F. Mescher, Division of Membrane Biology, Medical Biology Institute, La Jolla, CA 92037

Engagement of the TCR of T cells normally results in activation of phosphatidylinositol hydrolysis to yield diacylglycerol and inositol phosphate's (IP) second messengers. CTL can lyse some targets in the presence of EGTA. Under these conditions, release of IP's cannot be detected, but specific TCR-mediated signalling is clearly occurring [O'Rourke, A.M. and M.F. Mescher, *J. Biol. Chem.* **263**:18594 (1988)]. This finding indicated the existence of some additional TCR-mediated signalling pathway in CTL. We describe here that both target cell lysis by CTL and CTL degranulation in response to target cells or anti-TCR antibodies are inhibited by drugs which block arachidonic acid release from phospholipid. The drugs act at an early stage of the degranulation response, consistent with their acting on signalling events and not on the degranulation mechanism, *per se*. Furthermore, the drugs inhibit responses without affecting the levels of inositol phosphates produced in response to the stimuli. Labeled CTL release ^{3}H-AA upon interaction with targets, but further metabolites of AA cannot be detected. ^{3}H-AA release occurs rapidly, and is detected within 15 seconds of CTL interaction with targets. The results strongly suggest that activation of a phospholipase, probably PLA2, occurs upon perturbation of the TCR of CTL. The released AA, or possibly the lysophospholipid produced, appears to act as a critical second messenger in activating both cytolytic and degranulation responses.

F 237 REGULATION OF CD4 ASSOCIATED $p56^{LCK}$ BY ANTIBODY BINDING TO CD4 Andreani Odysseos, Mark Drotar and Christopher E. Rudd Division of Tumor Immunology, Dana-Farber Cancer Institute and Harvard Medical School

The CD4 and CD8 antigens can synergise with the T-cell receptor complex (Ti/CD3 complex) in the triggering of T-cell activation. Recently, we defined a new class of protein-tyrosine kinase receptor formed by the association of CD4 and CD8 with the src-related kinase $p56^{lck}$ (Rudd et al.,1988 PNAS 85,5190) which can readily phosphorylate the CD3 complex in vitro (Barber et al., 1989 PNAS 86,3277). We have extended this work by studying the effect of antibody binding to CD4 on $p56^{lck}$ associated activity. Antibody was bound to intact cells, membranes purified and the kinetics of $p56^{lck}$ autophosphorylation measured over 10 seconds to 7.5 minutes in the presence of inhibitors of phosphatases. Soluble anti-CD4 antibody (either crosslinked or uncrosslinked) caused a significant (2-5 fold) increase in the rate of the reaction over the initial 120 seconds. By 5 to 7.5 minutes, the reaction was saturated showing equivalent levels of phosphorylation in treated and untreated samples. Antibodies to various regions (E1 to E4 regions) of the CD4 antigen exerted this stimulatory effect. We have also recently demonstrated an interaction between the CD4:$p56^{lck}$ and Ti/CD3 complexes (see accompanying abstract, Poster # F240). The relationship between the stimulatory effects of anti-CD4 antibodies and the interaction between CD4:$p56^{lck}$ and Ti/CD3 will be discussed.

F 238 CALCIUM-DEPENDENT PROTEOLYSIS OF LIPOCORTIN I BY AN ENDOGENOUS A431 CELL MEMBRANE PROTEASE, Catherine J. Pallen and Siew Yeam Chuah, Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511. When A431 cell membranes were used to phosphorylate purified placental lipocortin I, proteolysis of lipocortin was observed. Cleavage of the 38 kDa phosphotyrosyl-lipocortin I generated a truncated but still phosphorylated form of lipocortin, indicating that proteolysis occurred at a site N-terminal to the site of tyrosine phosphorylation. Both phospho- and nonphospho-lipocortins could be cleaved by the membrane protease, but prephosphorylation enhanced the rate of proteolysis 2-fold during the initial reaction and by 60 min almost half of the phospho-lipocortin was cleaved. Proteolysis was calcium-dependent but a variety of calcium-dependent protease inhibitors were unable to block cleavage, suggesting that this calcium effect was due to the calcium-binding properties of lipocortin I itself. Proteolysis was temperature-dependent, occurring at 30°C and not at 0°C. The responsible protease is A431 cell membrane-associated as no similar cleavage was observed if synthetic phospholipid liposomes or inside-out erythrocyte membranes were substituted for A431 cell membranes. The ability of an endogenous membrane protease to catalyze this specific cleavage in a calcium-dependent manner indicates that this event may occur in the cell where it could have important effects on the functional properties of lipocortin I.

F 239 CSF-1 induces monocytic differentiation and chemotaxis of IL-3 dependent cells transfected with the human CSF-1 receptor. J. H. Pierce, G. Cox, E. DiMarco, P.P. Di Fiore, D.Lombardi, P. Anklesaria, L. Varesio, A. Sachaguchi and S.A. Aaronson. LCMB, NCI, Bethesda, MD. Expression vectors containing normal or point mutated human c-*fms*/CSF-1 receptor genes were transfected into IL-3-dependent 32D cells in order to determine the effects of CSF-1 signalling in this clonal myeloid progenitor cell line. CSF-1 triggered mitogenicity, long-term growth, and synchronous monocyte differentiation of the 32D-c-*fms* cells. CSF-1-induced monocytic differentiation could be reversed by IL-3, implying that signals generated by CSF-1 did not evoke irreversible commitment to a terminally differentiated macrophage. CSF-1 was also found to be a potent chemoattractant for 32D-c-*fms* cells suggesting that CSF-1 may serve to recruit monocytes from the circulation to tissue sites of inflammation or injury. While c-*fms* overexpression did not release the 32D cells from factor dependence, expression of the point mutated c-*fms* protein, which possessesconstitutive tyrosine kinase activity, was able to abrogate their IL-3 dependence and induce tumorigenicity. These cells also acquired a monocyte phenotype, implying that partial monocytic differentiation does not interfere with progression of a cell to a malignant state. These results suggest that if c-*fms* contributes to the development of human myeloid leukemia, it does so through its constitutive activation by an autocrine or paracrine mechanism or by genetic aberrations in c-*fms*, itself.

F 240 IDENTIFICATION OF A PHYSICAL ASSOCIATION BETWEEN THE CD4/CD8:p56^{LCK} AND Ti (TcR)/CD3 COMPLEXES Christopher E. Rudd, Kristine Burgess, Craig Zalvan and Andreani Odysseos Division of Tumor Immunology, Dana-Farber Cancer Institute and Harvard Medical School
The role of the CD4 and CD8 antigens as accessory structures in the recognition of foreign antigen by the Ti(TcR)/CD3 complex has implied that the possible simultaneous formation of a physical interaction between CD4/CD8 and the TcR complex. Recently, we defined a new class of protein-tyrosine kinase receptor formed by the interaction of CD4/CD8 with the src-related kinase p56^{lck} (Rudd et al.,1988 PNAS 85,5190) which can readily phosphorylate the CD3 complex in vitro (Barber et al., 1989 PNAS 86,3277). In this study, we have taken advantage of the sensitivity of CD3 labelling in the phosphotransferase assay to demonstrate a direct physical linkage between the CD4/CD8:p56lck and Ti(TcR)/CD3 complexes. Immunopurification analysis and the labelling of purified membrane vesicles with ^{32}P-ATP revealed the labelling of several bands at 55-70Kd, 40-42Kd, 21-26Kd and 14-20Kd. Significantly, antibodies to CD4, CD8 and p56^{lck} precipitated a complex formed of CD4:p56lck or CD8:p56^{lck} linked to the CD3 γ ε and ζ chains. Digitonin extraction further preserved the CD4/CD8:p56lck:CD3 complex which appears to exist as a pre-formed complex on transformed T cells in the apparent absence of presenting cells. The identification of a novel sub-complex comprised of CD4/CD8:p56^{lck} and CD3 is likely to be important in T-cell activation and to our understanding of the components involved in the regulation of p56^{lck} activity.

F 241 A COMMON MOTIF IN THE CYTOPLASMIC TAIL OF CD4 AND CD8 MEDIATE THEIR BINDING TO THE SAME DOMAIN OF P56^{lck}. Andrey S. Shaw[1], Jan Chalupny[2], Kurt E. Amrein[3], J. Andrew Whitney[4], Paula Kavathas[5], Bartholomew M. Sefton[3], and John K. Rose[1,4], Departments of Pathology[1], Immunobiology[2], Cell Biology[4], and Laboratory Medicine[5], Yale University School of Medicine, New Haven, CT 06510; Molecular Biology and Virology Laboratory[3], Salk Institute, San Diego, CA 92138.

The association of the T cell glycoproteins, CD4 and CD8, with the tyrosine protein kinase, p56^{lck}, is likely to play an important role in signal transduction during T cell development and activation. Because CD4 and CD8 do not exhibit any obvious protein similarity and because CD4 and CD8 may transduce distinct signals, we were interested in mapping the interacting domains. Using site-directed mutagenesis to generate a series of deleted and chimeric mutant proteins, we have determined that a eight or nine amino acid motif present in the cytoplasmic tails of CD4 and CD8A but not CD8B1 or CD8B2 is both necessary and sufficient for binding to p56^{lck}. Furthermore, we have determined that only two of the eight residues (both cysteines) are critical for the interaction. We also generated a series of p56^{lck} mutant proteins that have enabled us to determine that CD4 and CD8A bind to the same domain in the amino-terminus of p56^{lck}. This domain is notable for two cysteines which are both required for binding. These findings have implications both for the structure of the protein complexes and for CD4/CD8 signalling.

F 242 CHARACTERIZATION OF A NOVEL PUTATIVE RECEPTOR FOR A MEMBER OF THE INSULIN FAMILY, Peter Shier and Valerie M. Watt, Department of Physiology, University of Toronto, Toronto, Ontario, Canada, M5S 1A8 We have identified a new member of the mammalian insulin receptor (IR) family, the IR-related receptor (IRR). Nucleotide sequence analysis of human and guinea pig genomic DNA encoding IRR revealed that the gene contains 22 exons analogous in number, organization, and size to those of the human IR gene. The predicted structure of IRR includes all domains present in the IR and the insulin-like growth factor I receptor (IGF-IR) including the signal peptide, cysteine-rich, transmembrane and tyrosine kinase domains, as well as a putative processing site giving rise to two subunits. Indeed, the deduced protein product of the IRR gene is as closely related to the IR and to the IGF-IR as they are to each other, being maximal in the tyrosine kinase domains, where the three proteins exhibit an amino acid identity of approximately 80%. Southern blot and sequence analyses of guinea pig, human and rat genomic DNA showed that the IRR gene is highly conserved among mammals. Analyses of the sequence and chromosome localization of this IR-related receptor DNA in the human genome confirmed that this new receptor is distinct from the known receptors for insulin, IGF-I, and IGF-II, as well as from the other known IR family members (*ltk*, c-*ros*, *met*, and *trk*). We are currently analyzing the gene's promoter and assessing the extent of tissue-specific expression. In addition, we are probing the structural basis for its function to determine if IRR is a novel receptor for insulin, IGF-I, IGF-II, or an as yet unidentified peptide hormone or growth factor.

F 243 A LEUKEMOGENIC FORM OF *ERB*B GAINS THE ABILITY TO TRANSFORM FIBROBLASTS FOLLOWING INTRODUCTION OF A SINGLE POINT MUTATION WITHIN ITS TYROSINE KINASE DOMAIN. Hui-Kuo G. Shu[1], Robert J. Pelley[2], Thomas H. Carter[2] and Hsing-Jien Kung[1]. [1]Department of Molecular Biology and Microbiology and [2]Department of Medicine, Division of Hematology/Oncology, Case Western Reserve University, Cleveland, Ohio 44106.

Truncation of the N-terminal, ligand-binding domain of the *erb*B product, a putative avian growth factor receptor homologous to epidermal growth factor (EGF) receptor, results in a molecule capable of inducing erythroleukemia but not fibrosarcomas. Variants of this oncogene product display transforming potential with an expanded range of target tissues. Previously described variants that are capable of sarcoma formation contain extensive truncations or internal deletions of the C-terminal domain. We have now shown that a single point mutation within the centrally located tyrosine kinase domain permits transformation of fibroblasts in vitro and production of sarcomas in vivo. A higher autophosphorylation level in this molecule suggested that increased kinase activity may be responsible for sarcomagenic ability. The implicated mutation substitutes an isoleucine for valine at a site that is highly conserved among tyrosine and serine/threonine kinases and has been postulated to be part of the kinase's ATP-binding pocket. This apparently subtle mutation may therefore provide insight into the biochemical mechanism of protein kinases in general.

F 244 MULTIPLE MECHANISMS OF C-RAF ACTIVATION IN T-LYMPHOCYTES: PKC AND NON-PK MEDIATED, Jeffrey N. Siegel, Ulf Rapp, Richard D. Klausner and Lawrence E. Samelson.

Ligand binding to the T cell receptor (TCR) causes the activation of two distinct kinase pathways: protein kinase C (PKC) and an unknown tyrosine kinase. In order to define the more distal events in activation, we studied changes in the cytosolic serine/threonine kinase C-raf in the T cell hybridoma 2B4. To study T cell activation in the absence of antigen presenting cells (APC), we used phorbol ester and the stimulatory anti-Thy 1 antibody G7, which activates the same two kinase pathways as TCR-mediated signalling. In earlier studies, we found that both agents caused hyperphosphorylation of c-raf and an increase in kinase activity in a PKC dependent manner. In order to measure TCR-mediated signalling requiring an APC, we stimulated 2B4 cells with anti-TCR MAb presented on a B cell tumor (LK). Anti-TCR stimulation caused hyperphosphorylation of c-raf exclusively on serine. In addition, we found that c-raf kinase activity was increased in a dose-dependent manner. Surprisingly, we also found that addition of LK cells alone increased T-cell c-raf kinase activity reproducibly though to a small degree. Furthermore, PMA, at a dose which increased c-raf kinase activity minimally, synergized with B cells to cause a substantial increase. C-raf kinase activity contributed by the LK cells was minimal with or without PMA. Of note, when the T cells were depleted of PKC, the addition of LK cells caused a 15-fold increase in the T cell kinase activity, comparable to that seen in control T cells stimulated with a high dose of PMA, suggesting that the effect of LK cells is not only PKC independent but may actually be inhibited by PKC in resting cells. This effect of LK cells is not mediated through the TCR as TCR-blocking antibodies do not diminish the effect. We conclude that when T cells are stimulated by ligand on accessory cells, in addition to the TCR mediated signal, an additional signal is induced by the accessory cells which can activate T cell c-raf by a mechanism which is distinct from PKC and potentially synergistic.

F 245 RAPID RELEASE OF GLUCOSE 6-PHOSPHATE DEHYDROGENASE (G6PD) BY PLATELET-DERIVED GROWTH FACTOR (PDGF) AND EPIDERMAL GROWTH FACTOR (EGF) IN PERMEABILIZED CELLS. Robert C. Stanton and Lewis C. Cantley. Harvard Medical School and Tufts University. Boston, MA. Hexose monophosphate shunt (HMP) activation is an important step in cell division, providing ribose 5-phosphate for DNA synthesis. We have previously shown that EGF and PDGF increase activity of the rate limiting enzyme, G6PD, within 1 min. To determine the activation mechanism, rat renal cortical cells were permeabilized with streptolysin O and activity of G6PD was measured spectrophotometrically in supernatants from permeabilized cells. Permeablized, but unstimulated cells released < 1% of total cell G6PD activity. Within 1 min. of addition to permeabilized cells, EGF (10 nM) and PDGF (1 nM) caused release of 11.16 +/- 1.7% and 10.0 +/- 2.16% of total cell G6PD activity, respectively. The non-hydrolyzable analog of GTP, GTP γ S (18μM), caused a similar release of G6PD. Preincubation of the permeabilized cells with GDPβS (500 μM) prevented the release caused by PDGF and GTPγS, but did not inhibit the EGF response. Neither phorbol myristate acetate (100 nM) nor inositol triphosphate (20 uM) stimulated release. These results suggest that G6PD is bound to some structural component of the cell; and that EGF and PDGF cause release of this intracellularly bound G6PD into the cytosol.

F 246 CELLULAR SUBSTRATES FOR THE T LYMPHOCYTE-SPECIFIC PROTEIN TYROSINE KINASE p56^{LCK}. Peter A. Thompson[1,2], Ulf R. Rapp[3], Sue Goo Rhee[4], Jeffrey A. Ledbetter[5], Joseph B. Bolen[1], Laboratory of Tumor Virus Biology[1], NCI-Navy Medical Oncology Branch[2], National Cancer Institute, Laboratory of Biochemistry, National Heart, Lung, and Blood Institute[3], National Institutes of Health, Bethesda, Maryland 20892, Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, Maryland 21701[3], Oncogen[5], 3005 First Avenue, Seattle Washington 98121

The src-related protein tyrosine kinase p56^{lck} is abundantly expressed in normal human T lymphocytes. Recent work has shown that this tyrosine kinase associates non-covalently with the CD4, and CD8 surface antigens in T lymphocytes to form stable heterodimers. Antibody-mediated cross-linking of cell surface CD4 results in the rapid stimulation of the enzymatic activity of the associated p56^{lck}, and the appearance of novel phosphotyrosine containing proteins on antiphosphotyrosine immunoblots of whole cell lysates. Previous results from our laboratory have identified two of these candidate substrates for p56^{lck}; a 56kd protein which is p56^{lck}, and a 21kd protein which is the ζ subunit of the T-cell antigen receptor complex. We now present evidence that a 70-72kd protein which is phosphorylated on tyrosine following antibody-mediated cross-linking of cell surface CD4 is the cytoplasmic serine-threonine kinase p70^{raf}. The effect of this phosphorylation on the enzymatic activity of p70^{raf} will be discussed.

F 247 A CD45-RELATED cDNA FROM NIH 3T3 CELLS
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cDNAs from a variety of sources were examined for the presence of protein tyrosine phosphatase (PTPase)-specific sequences. Synthetic oligonucleotide primers corresponding to regions conserved in the catalytic domains of several known PTPases were used to amplify homologous domains by the polymerase chain reaction. Amplification of NIH 3T3 cDNAs produced a fragment of the predicted size. This fragment was cloned and sequenced. The predicted amino acid sequence over 33 residues was 66% homologous to mouse CD45, 51% homologous to human LAR and 42% homologous to human PTPase 1B. This fragment was used as a probe to screen an NIH 3T3 cDNA library. Several positive clones have been isolated and are currently being characterized.

F 248 CYTOKINE REGULATION OF CD45 PHOSPHORYLATION AND TYROSINE KINASE ACTIVITY IN MURINE T LYMPHOCYTES, Mary A. Valentine#, Jeffrey A. Ledbetter*, Fran Pinault^, David L. Brautigen^, Robert Voice+, Michael Widmer+, Edward A. Clark# and Byron Gallis+. Genetic Systems*, Section of Biochemistry, Brown University ^, Immunex+, and Department of Microbiology#, University of Washington; Seattle, Washington, 98195.

Lymphocytes express the CD45 protein on their surface, a molecule recently shown to act as a tyrosine phosphatase in both T and B lymphocytes. Several cytokines that regulate activation have been reported to affect protein phosphorylation. Therefore, we tested if IL-2 could alter phosphorylation of the CD45 molecule. Two dimensional phosphopeptide maps clearly showed increased phosphorylation of CD45 on at least two unique peptides. We next asked if addition of IL-2 might also alter the tyrosine phosphatase activity of CD45 under these conditions. We incubated murine IL-4-dependent cytotoxic T cells (CTL-L) with medium or IL-2 and assayed CD45 tyrosine phosphatase activity on a phosphotyrosine phosphorylated substrate. Tyrosine phosphatase activity was altered concurrently with increased CD45 phosphorylation. Addition of IL-2 did not change surface expression of CD45 as measured by FACS analysis. These observations are extended into a model in which kinase activation initiated through the IL-2 receptor might regulate tyrosine phosphatase activity. (Supported by NIH grant PO1 GM42508 and Immunex Corporation.)

F 249 THE P210 *bcr/abl* TYROSINE KINASE CAUSES CHRONIC MYELOGENOUS LEUKEMIA IN MICE. R.A. Van Etten, G.Q. Daley, and D. Baltimore. Whitehead Institute for Biomedical Research and Department of Biology, M.I.T., Cambridge, MA 02139.

Tumor cells from human patients with the myeloproliferative disease chronic myelogenous leukemia (CML) express the P210 *bcr/abl* protein, which is a dysregulated non-receptor tyrosine kinase. To determine whether P210 *bcr/abl* is capable of inducing CML, murine bone marrow was infected with a retrovirus encoding P210 *bcr/abl* and adoptively transferred into irradiated syngeneic recipients. Transplant recipients developed a variety of hematological malignancies, most prominently a myeloproliferative syndrome with a striking resemblence to human CML. These mice had approximately single copy levels of the retroviral provirus in bone marrow, spleen, and peripheral blood cells at autopsy, and expression of the P210 protein in early myeloid cells was demonstrated by an immunofluorescence assay. The provirus was passed to day 14 spleen colonies in an adoptive transplant, indicating that an early pluripotent cell similar to CFU-S was infected. Thus, the P210 tyrosine kinase, expressed within the hematopoietic system, causes an expansion of the myeloid compartment with preservation of terminal differentiation.

F 250 Regulation of Epidermal Growth Factor Receptor Signal Transduction: Role of Gangliosides, Frances M. B. Weis and Roger J. Davis, Department of Biochemistry, University of Massachusetts Medical School.

The addition of gangliosides to tissue culture cells causes a decrease in the tyrosine protein kinase activity of the epidermal growth factor (EGF) receptor and an inhibition of EGF -stimulated growth. Based on these data, the hypothesis that the EGF receptor is physiologically regulated by gangliosides has been proposed by Bremer E.G., Schlessinger J., & Hakomori S. (J. Biol. Chem. **261** 2434-2440; 1986). To test this hypothesis, a mutant Chinese hamster ovary cell line (clone *ldl* D) that has a reversible defect in the biosynthesis of gangliosides (Kingsley, D.M., Kozarsky, K.F., Hobbie, L. & Krieger, M., Cell **44** 749-759; 1986) was investigated. The human EGF receptor cDNA was expressed in the mutant cells and the properties of the EGF receptor were examined using cells grown under permissive and non-permissive conditions. Changes in ganglioside expression were not observed to cause any significant alterations to the affinity or number of EGF receptors detected at the cell surface. However, decreased levels of ganglioside expression were associated with: 1) increased EGF receptor autophosphorylation on tyrosine residues and, 2) increased EGF -stimulated cellular proliferation. The inverse correlation observed between the level of ganglioside expression and signal transduction by the EGF receptor is consistent with the hypothesis that the function of the EGF receptor is physiologically regulated by gangliosides.

F 251 LIGAND-INDUCED TRANSFORMATION BY A NON-INTERNALIZING EGF RECEPTOR. Alan Wells*, John B. Welsh*, Gordon N. Gill#, and Michael G. Rosenfeld#; Depts. of *Pathology and #Medicine, University of California, San Diego, CA 92093.

Activation of the EGF receptor initiates a cascade of cellular events. Upon binding of ligand the intrinsic tyrosine kinase is triggered, immediately followed by a rise in cytosolic free calcium concentration and receptor internalization and degradation. Increased mRNA transcription subsequently occurs, with prolonged stimulation leading to DNA synthesis and morphologic transformation. Identification of an EGF receptor mutant that fails to undergo down-regulation would permit investigation of the role which internalization plays in the mitogenic response. Removal of the carboxy-terminal 213 amino acids of the receptor, which contain the autophosphorylation sites, the calpain hinge and the newly-defined internalization region (Chen et al., Cell,1989, in press), resulted in such a molecule, while leaving the kinase activity intact. This mutant was expressed in NIH 3T3-derived NR6 cells, which are devoid of endogenous receptors. These cells responded to ligand similarly as cells carrying wild-type receptors, but were more sensitive at low levels of ligand. Augmentation of cell growth and anchorage independent growth was seen at concentrations which faied to elicit these responses in cells presenting the wild-type receptor. These findings imply that activation of the kinase activity at the plasma membrane is fully sufficient for the growth enhancing effects of EGF, and that down-regulation serves as an attenuation mechanism without which transformation ensues.

Serine Threonine Protein Kinases and Mechanisms Utilized by Single Cell Organisms

F 300 PHOSPHATIDYLINOSITOL-3 KINASE IS PRESENT IN THE YEAST *SACCHAROMYCES CEREVISIAE*, Kurt R. Auger, Christopher L. Carpenter, Lyuba Varticovski and Lewis C. Cantley, Department of Physiology, Tufts University School of Medicine, Boston, MA 02111.

The metabolism of polyphosphoinositides has been shown to be an important factor in controlling the proliferation of *Saccharomyces cerevisiae*. The monophosphate form of phosphatidylinositol has been assumed to be phosphatidylinositol-4-phosphate (PI-4-P). Evidence from our laboratory has established that a phosphatidylinositol (PI) kinase which phosphorylates the D-3 position of the inositol ring (PI-3 kinase), is associated with many activated protein-tyrosine kinases and may play an important role in the signalling of cell proliferation (Auger et al. Cell 57, 167-175, 1989). To determine the evolutionary conservation of this enzymatic activity, we investigated its presence in yeast. *In vitro* PI kinase assays of yeast cell homogenates demonstrated that PI-3 kinase activity was present. Preliminary biochemical characterization of the activity suggested that it was quite different from the mammalian enzyme yet catalyzed the same reaction, i.e. phosphorylating the D-3 hydroxyl position of the inositol ring of phosphatidyl-myo-inositol. The conservation of this enzymatic activity from yeast to man suggests that it has an important functional role in the cell cycle.

F 301 REGULATION OF SIGNAL TRANSDUCING PROTEIN-SERINE/ THREONINE KINASES; pp70-S6 KINASE, pp90-S6 KINASE II AND S6 KINASE II - PROTEIN KINASE BY GROWTH FACTORS AND ONCOGENES.

John Blenis, Rey-Huei Chen and Jongkyeong Chung, Department of Cellular and Molecular Physiology, Harvard Medical School, 25 Shattuck St., Boston, MA. 02115.

We are presently characterizing the regulation of three distinct mitogen- and oncogene-activated protein-serine/threonine kinases. Two of these were identified by their ability to phosphorylate 40S ribosomal protein S6 in vitro, pp70-S6 protein kinase (pp70-S6K) and pp90-S6 protein kinase II (pp90-S6KII). The third was identified by its ability to phosphorylate recombinant S6KII in vitro (S6KII-PK). S6KII-PK and pp90-S6KII are maximally activated by 2 min following the addition of serum growth factors to quiescent Swiss 3T3 cells. pp70-S6K is maximally activated by 10 min. Every mitogen tested rapidly activates these enzymes via post-translational protein phosphorylation mechanisms. During the G_0/G_1 transition, pp90-S6KII and S6KII-PK activities are coordinately regulated, independently of the regulation of pp70-S6K, suggesting that the growth-modulating signalling pathways have already diverged. Futhermore, these signal transducing protein kinases are regulated by protein kinase C - independent and - dependent mechanisms. The identification and characterization of growth signalling protein phosphorylation cascades should continue to improve our understanding of the processes regulating normal and neoplastic cell growth.

F 302 DIFFERENTIAL EXPRESSION OF PROTEIN KINASE C-ISOFORMS DURING MULTISTEP CARCINOGENESIS

Christoph M.B. Borner, Sarah F. Nichols, Ling Ling Hsieh, W.-L.W. Hsiao and I. Bernard Weinstein. Institute of Cancer Research, Columbia University, New York, NY 10032

We established rat embryo fibroblast and rat liver epithelial cell lines which gradually progress to a fully tumorigenic phenotype by introducing stepwise PKCßI cDNA and the activated c-H-ras (T24) oncogene. The overexpressed PKCßI (79-kDa) and a newly discovered higher molecular weight (84-kDa) PKCßI-related protein were more membrane-associated in the fully transformed PKCßI plus H-ras cells than in the PKCßI cells suggesting greater activation of PKC in the former cells. Unexpectingly, the endogeneous PKCδ form was 6-10 fold induced and the endogeneous PKCε form 5-7 fold decreased in the PKCßI plus H-ras tumorigenic cells, when compared to the normal parental cells. These changes in the expression of endogeneous PKC genes were demonstrated at both the protein (Western blot analysis using isoform-specific antibodies) and the mRNA (Northern blot analysis) levels. These data provide evidence that PKCßI and an activated ras oncoprotein can act cooperatively to cause cell transformation and that this is associated with alterations in the expression of other isoforms of PKC endogeneous to these cells. Thus, alterations in the expression of various isoforms of PKC may play a role in tumorigenesis.

F 303 p40tax TRANSACTIVATES HTLV-I IN HUMAN T CELLS THROUGH A PK-C DEPENDENT PATHWAY. Anna T.Brini, David Kelvin and William L. Farrar. Laboratory of Molecular Immunoregulation, BRMP, NCI-FCRF, Frederick, MD 21701-1013.

HTLV-I, the etiological agent of adult T-cell leukemia, encodes a transcriptional activator, p40tax, that increases transcription of HTLV-I and of other cellular genes. The p40tax inducible enhancer has been localized to three copies of a 21 base pair repeat within the HTLV-I LTR. Here we examine different signal transduction pathways involved in HTLV-I activation by p40tax. We cotransfected a HTLV-I CAT plasmid with a p40tax expression vector or one of its mutant form in human T cells. HTLV-I is induced by p40tax, by PMA, and by forskolin. Two synthetic genes that code for an active fragment of the protein inhibitor of the cAMP-dependent protein kinase (PKI) and for an inactive form were transfected in T cells with the HTLV-I LTR CAT construct and the p40tax expression plasmid. PKI specifically inhibits forskolin induction with no effect on the PMA and on the p40tax activation of HTLV-I CAT. In contrast, transfection of HTLV-I CAT and p40tax in protein kinase C-depleted T cells completely blocks HTLV-I activation by p40tax and PMA, with no effect on the adenyl cyclase pathway. The same result was obtained when HTLV-I transfected T cells were treated with the protein kinase C inhibitor H-7. We hypothesize that HTLV-I activation by p40tax involves the protein kinase C pathway.

F 304 PURIFICATION AND PROPERTIES OF A DNA-DEPENDENT PROTEIN KINASE FROM HELA CELL NUCLEI, Tim Carter, Ivon Sun, Ivana Vančurová, Susan DeLeon and Willard Lou, Department of Biological Sciences, St. John's University, Jamaica, NY 11439.
The endpoint of signal transduction pathways mediated by protein kinases is frequently the regulation of nuclear processes. Identification of protein kinases that are activated within the nucleus is therefore likely to contribute to understanding such regulation. We are studying a novel protein kinase (PKD) from HeLa cell nuclei that requires double-stranded (ds) DNA for phosphorylation of a variety of substrates *in vivo* and *in vitro*. PKD has been purified ~5000-fold, and is a serine-threonine casein kinase most active with Mg^{2+} and ATP, but the enzyme is weakly activated by Mn^{2+}, and can utilize GTP as a phosphate donor. DNA-dependent activity is salt-sensitive, and also inhibited by polyamines, heparin, and N-ethylmaleimide. Highly purified PKD contains a major, autophosphorylated, polypeptide of M_r = ~300 kDa by SDS-PAGE, similar in size to the native enzyme, and binds to DNA in gel shift studies. Preliminary results using monoclonal antibodies on western blots and in enzyme inhibition experiments are consistent with identification of the 300 kDa polypeptide as an integral component of the enzyme.

F 305 ZINC AS A POSSIBLE MEDIATOR OF SIGNAL TRANSDUCTION IN T LYMPHOCYTE ACTIVATION
Péter Csermely and János Somogyi, Institute of Biochemistry I., Semmelweis University School of Medicine, PO Box 260, Budapest, H-1444 Hungary

Increasing interest is focused on the role of zinc in biological systems. A rapidly growing family of proteins --including protein kinase C-- contains "zinc fingers". On the other hand zinc is able to displace calcium from a number of Ca-binding sites and in this way zinc may modify calcium-mediated cellular processes. Our experimental data indicate that
- -- activation of protein kinase C induces the translocation of zinc from the nucleus to the cytosol and plasma membrane
- -- zinc activates cytosolic protein kinase C and increases its affinity towards phorbol esters at nanomolar free concentration
- -- zinc contributes to the binding of protein kinase C to plasma membrane and to desoxiribonucleic acid
- -- intracellular heavy metals (most probably: zinc) can diminish the Ca-ionophore-induced Ca-transport in T lymphocytes after the activation of protein kinase C.

These observations suggest that changes in intracellular zinc concentration may occur during the activation of T lymphocytes and, in turn, zinc may modify the calcium- and protein kinase C-dependent pathways of T lymphocyte activation. Experiments which directly address these questions are in progress in our laboratory.

F 306 IDENTIFICATION AND PARTIAL SEQUENCE ANALYSIS OF THE MITOGEN/ ONCOGENE-ACTIVATED S6 KINASE, Stefano Ferrari and George Thomas, Friedrich Miescher Institute, CH-4002 Basel, Switzerland.
Addition of growth factors to quiescent Swiss cells results in the ordered phosphorylation of five serines at the C-terminus of 40S ribosomal protein S6. We have purified the kinase responsible for regulating this process in mitogen/oncogene-activated cells and from the liver of rats injected with cycloheximide. The two kinases proved to be identical in (A) molecular weight, 70 kd, (B) sensitivity to phosphatase 2A, (C) specific activity towards S6, ≈1 μmol/min/mg, (D) inhibition by μM Mn^{2+} in the presence of mM $MgCl_2$, and (E) phosphopeptide maps of autophosphorylated enzymes. That this is the kinase responsible for S6 phosphorylation in vivo is indicated by the fact that the activity of the enzyme increases in parallel with S6 phosphorylation in vivo, that at each step of the purification it acts as a single entity, and that it incorporates 4 to 5 moles of phosphate in vitro into the same S6 sites as observed in vivo. Sequence analysis of the N-terminus and of an internal CNBr fragment shows no significant homology to any known kinase, suggesting that this is a novel threonine-serine kinase.

F 307 REGULATION BY CYCLIC AMP OF ENDOTHELIAL SECRETION OF TISSUE-TYPE PLASMINOGEN ACTIVATOR AND ITS RAPID INHIBITOR, Robert B. Francis Jr., and Sara Neely, Department of Medicine, University of Southern California School of Medicine, Los Angeles, CA 90033. We investigated the effect of agents which raise intracellular cyclic AMP (cAMP) on the secretion of tissue-type plasminogen activator (t-PA) and type 1 plasminogen activator inhibitor (PAI-1) by cultured human umbilical vein endothelial cells (HUVEC). HUVEC monolayers were exposed to various concentrations of these agents in serum-free growth medium for 48 hours, and t-PA and PAI-1 measured in the supernate at 24 and 48 hours by ELISA. Significant inhibition of baseline (unstimulated) t-PA and PAI-1 secretion was observed with cholera enterotoxin, 1-methyl-3-isobutylxanthine (MIX), dibutyryl cAMP, prostaglandin E1 (PGE1), the stable prostacylin analogue iloprost, and arachidonic acid, which is converted by HUVEC to prostacyclin. Cholera toxin, MIX, dibutyryl cAMP, and PGE1 also significantly reduced or abolished the stimulation of t-PA release by thrombin and histamine, and, with the exception of MIX, also significantly reduced or abolished the stimulation of PAI-1 release by thrombin. MIX at a concentration below that required, when tested alone, to inhibit t-PA and PAI-1 secretion, significantly increased the inhibition of t-PA and PAI-1 secretion by cholera toxin, dibutyryl cAMP, and PGE1. We conclude that elevated cAMP inhibits HUVEC secretion of t-PA and PAI-1, both spontaneously and in response to agents which stimulate endothelial phosphoinositide pathway activity (thrombin and histamine).

F 308 *daf-1*, A GENE INVOLVED IN *Caenorhabditis elegans* DAUER LARVA DEVELOPMENT, ENCODES A NOVEL MEMBRANE PROTEIN KINASE, Laura L. Georgi, Patrice S. Albert and Donald L Riddle, Division of Biological Sciences, University of Missouri, Columbia, MO 65211
The dauer larva is a developmentally-arrested, non-feeding dispersal stage normally formed in response to overcrowding and limited food. More than twenty-five genes affecting dauer larva development have been identified. Nineteen of these genes have been ordered relative to each other based on epistatic relationships. The resulting hierarchy of genes is thought to specify a pathway for neural transduction of environmental cues. The *daf-1* gene, which specifies an intermediate step in the genetic pathway, has been cloned by Tc1 transposon tagging. A *daf-1* probe detects a 2.5 kb mRNA of low abundance. The gene spans almost 6 kb of genomic DNA and contains eight introns ranging in size from 45 to 1379 bp. The DNA sequence predicts a 669-amino acid (74 kD) gene product containing stretches of hydrophobic residues characteristic of transmembrane proteins in its amino terminal half, and the conserved residues diagnostic of serine-threonine protein kinases in its carboxyl terminal half. The protein sequence shows greatest similarity to the *raf* proto-oncogene family. Three separate mutant alleles, including the two transposon-insertion alleles used in cloning the gene, are altered in the sequence encoding the kinase domain. The mutants form dauer larvae constitutively at elevated temperatures (20-25°C), so kinase activity apparently is involved in the inhibition of dauer larva formation. The longest cDNA clone has as its 5' end the last six residues of a 22-base *C elegans trans*-spliced leader sequence, and the genomic sequence at this position is a consensus splice acceptor site, indicating that the mRNA is *trans*-spliced. The 3' untranslated region of the message is 461 bases long. The third intron contains multiple copies of an octamer, CTACAGTA, most of which are concentrated in an extended imperfect direct repeat of 115-117 bp, present in roughly 2.5 copies. This intron hybridizes to multiple genomic bands on Southern blots at reduced stringency, and might contain regulatory sequences.

F 309 A CALCIUM-UNRESPONSIVE, PHORBOL ESTER/PHOSPHOLIPID-ACTIVATED PROTEIN KINASE FROM PORCINE SPLEEN, Michael Gschwendt, Hanno Leibersperger and Friedrich Marks, Department of Biochemistry, German Cancer Research Center, Heidelberg, F.R.G.
The particulate fraction of porcine spleen contains a protein kinase which can be activated by phospholipid and the phorbol ester TPA but does not respond to phospholipid and calcium (Ca). The kinase can be partially purified and separated from Ca-responsive protein kinase C (PKC) by sequential chromatography on various adsorbents, such as Q-Sepharose, hydroxyapatite, phenyl Sepharose, heparin agarose and protamin agarose. The partially purified kinase has a molecular weight of 76 kDa (p76-kinase) and hence is somewhat smaller than the similarly behaving p82-kinase from mouse epidermis and spleen. Autophosphorylation of p76-kinase is at least 10 times stronger than that of PKC. The staurosporine-like protein kinase inhibitor K252a clearly differentiates between the Ca-unresponsive p76-kinase and Ca-responsive PKC (IC50 values differ by two orders of magnitude). This might indicate that a sequence, which is missing in Ca-unresponsive types of the PKC family, is essential for the suppression of the enzyme by staurosporine and staurosporine-like inhibitors. The pseudosubstrate of PKC inhibits both enzymes with equal potency.

F 310 ANALYSIS OF THE MITOGENIC SIGNAL TRANSDUCTION PATHWAYS INDUCED BY BRYOSTATINS AND LYMPHOKINES IN HUMAN B LYMPHOCYTES.
Graeme R. Guy, Ng Siew Bee and Chua Sook Peng ,Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Cres, SINGAPORE 0511.

Bryostatins are isolated from marine bryophytes and are potent activators of protein kinase C (in the pmol range). They have been shown to be antiproliferative agents in assays on transformed cell lines. We demonstrate that bryostatin 1 and 2 have moderate proliferative activity when used alone on B cells, however they show synergistic stimulation of proliferation when used with either of the following agents; the calcium ionophore, ionomycin, interleukin 2, interleukin 4, B cell growth factor (BCGF) and the novel calcium ionophore, thapsigargen. Bryostatin 1 will however down-regulate the proliferative effects of the phorbol ester tumour promoter, PMA. Biochemical analysis, including protein phosphorylation, binding competition and gene expression showed differences between bryostatins and PMA. The possible site of action of the bryostatins including the possibility of them binding to other isoforms of protein kinase C is discussed.

F 311 INVOLVEMENT OF PROTEIN KINASES A AND C IN NF-κB ACTIVATION BY TNF AND IL-1.
Hans-Peter Hohmann, Roland Remy, Roland Kolbeck and Adolphus P. G. M. van Loon, Central Research Units, F. Hoffmann-La Roche & Co AG, CH-4002 Basel, Switzerland.

The nuclear factor κB (NF-κB) plays a crucial role in the expression of several genes involved in the immune-response. A DNA-binding protein very similar or identical to NF-κB can be activated in many different cells by PMA, LPS, and by the tumor necrosis factors α and β (TNF) or interleukin-1 (IL1). We are interested in signal transduction pathways triggered by TNF and, thus, in the activation of NF-κB by TNF. In the promyelocytic cell line HL60, NF-κB is strongly induced by TNF within minutes of incubation, weakly and delayed by PMA, and not by IL1. In the pre-B cell line 70Z/3, NF-κB is not induced by TNF (no TNF receptors were detected), but is induced by IL1 and PMA to a similar extent and with similar and fast kinetics. Staurosporin, a potent protein kinase inhibitor, affecting e.g. protein kinases A and C, inhibited NF-κB activation by PMA both in HL60 and 70Z/3 cells and the IL1 induced activation of NF-κB in 70Z/3 cells. In contrast, TNF-induced activation of NF-κB in HL60 cells was not inhibited by staurosporin, but staurosporin treatment even increased the degree of NF-κB activation at low TNF concentrations. This suggested that protein kinase A (PKA) and, thus, cAMP is not necessarily involved in activation of NF-κB, in contrast to data presented by Shirakawa and Mizel (Mol. Cell. Biol. 9, 1989, 2442-2430). To test involvement of PKA and cAMP more extensively, forskolin, which stimulates ATP-cyclase, was used to increase the intracellular cAMP levels in both cells, but no or only marginal activation of NF-κB was observed. Inhibitors of phosphodiesterase also increased cellular cAMP levels, but again NF-κB activity was hardly detectable. In addition, TNF activated NF-κB, but did not increase the cellular cAMP content. We conclude, that activation of NF-κB by TNF is not mediated by cAMP and confirm the existence of at least two different pathways, which activate NF-κB. A protein kinase independent pathway transduce the TNF signal. An other pathway used by IL1 depends on functional protein kineses.

F 312 A RAPID METHOD FOR THE PURIFICATION OF A SERINE/THREONINE-SPECIFIC PHOSPHOPROTEIN PHOSPHATASE FROM THE BOVINE BRAIN: EFFECTS OF INOSITOL POLYPHOSPHATES ON ENZYME ACTIVITY.
Richard E. Honkanen, Jean Zwiller, Patrick J. Sousa, Stuart S. Nakamoto, Martha Y. Kanemitsu, Sharon L. Daily and Alton L. Boynton, Cancer Research Center of Hawaii, Honolulu HI, 96813. The reversible phosphorylation of proteins is now recognized to be of paramount importance in the regulation of many intracellular functions. Here we describe a rapid method for the purification of a phosphoprotein phosphatase to apparent homogeneity from bovine brain utilizing cholate extractable proteins obtained from a crude membrane preparation followed by heparin-Sepharose column chromatography and FPLC on Mono Q and Superose 6. The catalytic subunit of this brain phosphoprotein phosphatase best resembles a "Type 1" phosphatase in that its activity is inhibited by Inhibitor 2, and it has a preference for the B subunit of phosphorylase kinase. However, its intermediate affinity for okadaic acid (IC_{50}=3.2 nM) and the observation that its activity towards phosphorylase A is not effected by the addition of heparin suggest that this phosphatase may represent a subclass within the larger class of "Type 1" phosphatases. In addition, using both crude and lysine rich histone phosphorylated by protein kinase A as substrate, myo-inositol 1,4,5-trisphosphate (IP_3) and myo-inositol 1,3,4,5-tetrakisphosphate stimulate the release of phosphate in a dose dependent manner.

F 313 IDENTIFICATION OF A HUMAN DsDNA-ACTIVATED SERINE/THREONINE PROTEIN KINASE THAT PHOSPHORYLATES THE HEAT SHOCK PROTEIN, HSP90, AS WELL AS SV40 T-ANTIGEN, Susan P. Lees-Miller, Yuh-Ru Chen, and Carl W. Anderson, Biology Department, Brookhaven National Laboratory, Upton, NY 11973.

A serine/threonine protein kinase activity (dsDNA-kinase) has been purified approximately 1000-fold from HeLa cell extracts that is strongly activated by double-stranded DNA. Activation is specific for dsDNA but largely independent of DNA sequence; poly(dA-dT) and poly(dG-dC) activate as well as natural dsDNAs. Neither ssRNA or dsRNA activate the kinase, while ssDNA, phosphate, pyrophosphate, and heparin inhibit kinase activation. Apparent kinase binding constants (K_m) are 1µg/ml for calf thymus DNA and 24 µM for ATP; GTP is utilized poorly by the kinase. The dsDNA-kinase chromatographs during gel filtration in 1 M KCl as a protein of 350-kDa. Partially purified dsDNA-kinase contains polypeptides of 52-kDa, 65-kDa, 81-kDa, 110-kDa, and 350-kDa that are phosphorylated in a dsDNA-dependent manner. The only identified endogenous substrate is the heat shock protein, hsp90. DsDNA-dependent phosphorylation of hsp90 was previously observed in extracts prepared from widely divergent sources including rabbit reticulocytes, and frog, clam, or sea urchin oocytes or eggs (Walker et al., EMBO J. 4, 139-145, 1985). Of the two human hsp90 polypeptides (alpha and beta), only the alpha form is phosphorylated by the dsDNA-kinase. Phosphorylation occurs on two N-terminal threonine residues in the sequence PEETQTQDQPM-. The dsDNA-kinase also phosphorylates alpha and beta casein and phosvitin, but not BSA or the core histones. Recently, SV40 large T-antigen was found to be a good dsDNA-kinase substrate; identification of these phosphorylation sites is in progress. Research supported by the Office of Health and Environmental Research, U. S. Department of Energy.

F 314 FUNCTIONAL COMPONENTS OF THE PROTEIN KINASE C REGULATORY DOMAIN, NINA MILONA, BERTA STRULOVICI*, LISA SULTZMAN AND JOHN KNOPF, DEPARTMENT OF MOLECULAR BIOLOGY, GENETICS INSTITUTE INC., 87 CAMBRIDGEPARK DRIVE, CAMBRIDGE, MA 02140, *SYNTEX RESEARCH, 3401 HILLVIEW AVENUE, PALO ALTO, CA 94303. Protein Kinase C (PKC) has two 56 amino acid cysteine-rich regions which comprise the c-1 region of the PKC regulatory domain. Each of these 56 amino acid regions is shown to bind the phorbol ester [^{3}H]PDBu with approximately equal affinities. The natural activator of PKC, diacylglycerol, also competes with [^{3}H]PDBu for binding to each of these 56 amino acid regions. The role of the C-2 domain of PKC which also resides in the regulatory domain is unknown. We have been studying the regulation of PKC mutants which have had this region mutated or deleted. Our results suggest that this region is not involved in the calcium dependency of the enzyme but serves some other regulatory function.

F 315 PROTEIN KINASE C REGULATION OF AN EPIDERMAL GROWTH FACTOR - STIMULATED THREONINE PROTEIN KINASE. Ingrid C Northwood & Roger J. Davis, *Dept. of Biochemistry, University of MA Medical School, Worcester, MA 01655*

A protein kinase activity has been identified that phosphorylates the epidermal growth factor (EGF) receptor at threonine^{669} (J. L. Countaway, I.C. Northwood, and R.J. Davis ,1989 , J. Biol. Chem. **264** 10828-10835). An *in vitro* assay for this protein kinase using a synthetic peptide (T669) substrate was developed. Treatment of A431 cells with EGF and 4-phorbol 12-myristate 13-acetate (PMA) caused a 6- fold and a 2- fold increase in the protein kinase activity detected, respectively. A kinetic analysis of synthetic peptide T669 phosphorylation demonstrated that the increase in protein kinase activity observed was accounted for by an increase in V_{max}.

Previous studies have demonstrated that the treatment of A431 cells with PMA causes an inhibition of signal transduction by the EGF receptor. We therefore investigated the effect of PMA on the activation of the T669 protein kinase activity caused by incubation of cells with EGF. We observed that the extent of stimulation of peptide T669 protein kinase activity caused by EGF was not affected by pre- treatment of the cells with PMA. We conclude that the mechanism by which EGF stimulates the peptide T669 protein kinase activity is not subject to desensitization by phorbol ester.

F 316 12-O-TETRADECANOYLPHORBOL-13-ACETATE (TPA) CAUSES ALTERATIONS IN CELL CYCLE KINETICS AND STEROID HORMONE RECEPTOR GENE EXPRESSION IN LNCaP HUMAN PROSTATIC CARCINOMA CELLS. C.J. Ormandy, C.S.L. Lee, E.A. Musgrove and R.L. Sutherland. Garvan Institute of Medical Research, Darlinghurst, 2010, Sydney, Australia.

A major pathway of mitogenic signaling involves the Protein Kinase C (PKC) group of serine-threonine kinases. The phorbol ester TPA binds to PKC causing initial activation of phosphorylative activity followed by rapid depletion of PKC and the loss of PKC mediated effects. We have examined the effects of TPA on steroid hormone receptor gene expression and cell cycle kinetics to investigate the potential role of PKC in the control of LNCaP cell growth and differentiation.

LNCaP cells, grown in RPMI 1640-5% foetal calf serum were harvested 3 to 48 hours after treatment with TPA to deplete PKC. mRNA levels were examined by Northern blot analysis using specific cDNA probes for the human oestrogen (ER), progesterone (PR), glucocorticoid (GR), and 1,25 dihydroxyvitamin D3 (VDR) receptors. Flow cytometry was used to evaluate the cell cycle and morphological effects of TPA treatment. LNCaP cells responded in a dose- and time-dependent manner to TPA. Dose response experiments indicated a large decrease in proliferation rate between 0.1 and 10 nM TPA (ED50=400 pM at 48h) paralleled by a 50% loss of cells from the DNA synthetic phase and increased cellular size and granularity. Time course experiments using 10 nM TPA indicated that the alterations in cell cycle kinetics occured between 6 and 12 hours. Sex hormone receptor (PR, ER) mRNA levels were reduced by 20 % at 3 h, declined to 40% of controls by 24 h and remained suppressed at 48 h. Non sex steroid hormone receptors (GR, VDR) remained unchanged or increased slightly over this period.

These data indicate that TPA treatment results in a reduction of LNCaP proliferation rate and a decrease in the level of sex steroid hormone receptor mRNA. These observations suggest that PKC is a mediator of mitogenic signals and sex steroid hormone receptor gene expression in these cells.

F 317 INHIBITION OF PROTEIN KINASE C BY THE ANTICARCINOMA AGENT DEQUALINIUM.

S.A. Rotenberg, S. Smiley, M. Ueffing, R.S. Krauss, L.B. Chen, and I.B. Weinstein. Institute of Cancer Research, Columbia University, New York, NY 10032

Dequalinium has previously been shown to be an anticarcinoma agent (PNAS 84:5444-5448, 1987). The present study demonstrates that it can inhibit protein kinase C-β_1 isolated from an overproducing cell line with an IC50 = 8-15 μM. Further examination of the inhibition using structural analogues of dequalinium reveals specifc structure-activity relationships. Other studies show that the analogues display the same rank order of inhibitory potency when tested with the trypsin-generated catalytic fragment of the enzyme indicating that dequalinium inhibits kinase activity through an interaction with the catalytic subunit. Further studies argue that the ability of a given analogue to inhibit phosphotransferase activity correlates with its ability to compete with (^{3}H-)phorbol 12,13-dibutyrate binding on the intact enzyme (IC50 = 2-5 μM). This suggests that the inhibitor is either binding directly to the regulatory subunit as well or, that due to its interaction with the catalytic subunit, dequalinium produces an indirect effect on sites defined by phorbol ester binding. Kinetic analysis reveals that inhibition is noncompetitive with respect to ATP or phosphatidylserine. Studies conducted with types I, II, and III rat brain isozymes resolved by hydroxylapatite chromatography, demonstrate that dequalinium inhibits each of them with similar potency (IC50 = 11 μM) and imply that the site of contact on the enzyme is a highly conserved region. Studies of dequalinium with intact cells demonstrate that the inhibitor can protect control cells against phorbol ester-induced morphology changes but cannot protect PKC-overproducing cells, suggesting that an elevation in PKC levels alone is sufficient to overturn the protection conferred by dequalinium. On the basis of these results, we propose that protein kinase C could be a critical *in vivo* target of dequalinium. (Supported by NIH CA 02656, CA 19589-11, and CA 22427-10.)

F 318 SIMIAN VIRUS 40 LARGE T ANTIGEN INDUCES OR ACTIVATES A PROTEIN KINASE AS INDICATED BY ENHANCED PHOSPHORYLATION OF THE CELLULAR PROTEIN P53, Karl H. Scheidtmann (§) and Adolf Graessmann ($), (§) Institut für Immunbiologie Universität Freiburg, Stefan-Meier-Str. 8, D-7800 Freiburg, ($) Institut für Molekularbiologie, Freie Universität Berlin, Arnimallee 22, D-1000 Berlin 33, F.R.G. Alterations in the phosphorylation state of p53 from normal versus SV40-infected or transformed rat cells suggested that SV40 induces or activates a protein kinase one substrate of which is p53. Kinase induction is dependent on a functional, transformation-competent large T antigen whereas small T antigen is not required. Transformation-defective large T antigen mutants are impaired in kinase induction. Moreover, the kinase activity is greatly reduced in cells which have reverted to the normal phenotype due to a cellular mutation. These data suggest that the "p53-kinase" plays a role in SV40-induced transformation. The Kinase is associated with p53-immunoprecipitates and phosphorylates the same sites that are phosphorylated in vivo. Preliminary data suggest that this kinase does not correspond to the CDC2 kinase.

Supported by the Deutsche Forschungsgemeinschaft through SFB 31.

F 319 TUMOR NECROSIS FACTOR SIGNAL TRANSDUCTION: CELL-TYPE-SPECIFIC ACTIVATION AND TRANSLOCATION OF PROTEIN KINASE C, Stefan Schütze, Susanne Nottrott, Klaus Pfizenmaier, and Martin Krönke, Clinical Research Group of the Max-Planck-Society, 3400 Göttingen, F.R. Germany

We have investigated the changes in protein kinase C (PKC) activity following treatment of several cell lines with tumor necrosis factor (TNF). Binding-studies with [^{3}H]-PBt_2 on whole cells revealed rapid and transient activation of PKC in Jurkat, K562, and U937 cells with a maximum of phorbol ester-binding at 6 minutes after TNF-treatment. Upon subfractionation of TNF-treated U937 cells a transient increase of PBt_2-binding in the membrane fraction was accompanied by a long-term loss of PBt_2-binding in the cytosol, indicating a TNF-induced translocation of PKC from the cytosol to the cell membrane. Determination of the specific PKC-activity, using histone III-S as a substrate, revealed similar kinetics of PKC translocation in U937 cells. TNF also induced PKC-translocation in K562 and Jurkat cells. However, while TNF caused long-term downregulation of cytosolic PKC-activity in U937 cells, the cytosolic PKC-activity only transiently decreased in both Jurkat and K562 cells and then recovered to near basal levels. In the human nonmalignant fibroblast cell line CCD18, PKC was not activated by TNF. Together our data suggest that PKC-activation may play a major role in TNF signal transduction in some, but not all target cells.

F 320 ISOZYMES OF PROTEIN KINASE C CAN MODULATE THE DEGRADATION OF mRNAs HAVING AU DESTABILIZING SEQUENCES, Gray Shaw, Genetics Institute Inc., Cambridge, MA.

Previously we have shown that treatment of mammalian cells with protein kinase C (PKC) agonist such as PMA can cause the accumulation of mRNAs normally rapidly degraded due to AU-rich destabilizing sequences present in their 3' untranslated region. The mRNAs of this class include several lymphokines, cytokines and proto-oncogenes. In the experiments presented here, three independent CHO cell lines constitutively over expressing one of the cloned isozymes (beta, gamma or epsilon) of PKC have been analyzed to determine whether a particular isozyme is involved in this specific blockage of mRNA degradation. The preliminary results indicate that different isozymes of PKC may regulate the degradation pathway with varying efficiencies. Therefore the levels of a particular isozymes of PKC in a given cell type may dictate the responsiveness of that cell in its ability to block the mRNA degradation. This may explain why some cells types can modulate this specific mRNA degradation pathway more effectively than others.

F 321 PURIFICATION OF EARLY AND LATE PHASE S6 KINASE, Mira Šuša, Michel Siegmann and George Thomas, Friedich Miescher-Institut, P.O.Box 2543, CH-4002 Basel, Switzerland.

Earlier we showed that the S6 kinase from mitogen-stimulated Swiss mouse 3T3 cells is a 70 kD protein, which is activated by Ser/Thr phosphorylation (PNAS 85, 406 & 7154; 1988). More recently we found that the kinetics of EGF activation are biphasic, with the early phase peaking at 10 to 15 min and the late phase between 30 and 60 min. Down-regulation of protein kinase C has no effect on the early phase, but results in almost total loss of the late phase and a diminished mitogenic response (Cell 57, 817; 1989). Since a number of kinases have been implicated in S6 phosphorylation, we have purified the enzyme in both phases from ^{32}P-labelled cells. The results show that both kinases are equivalent to the mitogen-activated 70 kD kinase, each contains P-Ser and P-Thr and that they are differentially regulated by two distinct EGF-induced signalling pathways.

F 322 ANALYSIS OF RIBOSOMAL S6 PROTEIN KINASES (rsk) PRODUCED BY RECOMBINANT BACULOVIRUSES. T. A. Vik, T. J. Martins, and R. L. Erikson. Dept. of Cellular and Developmental Biology,Harvard University, Cambridge, MA 02138.The rapid phosphorylation of the ribosomal protein S6 is a highly conserved response of a variety of cells to mitogenic stimulation. In order to understand the regulation of the relevant enzymes, molecular clones for the S6 protein kinase have been obtained from multiple species. Two full length clones obtained from a Xenopus library encode proteins of 729 and 633 amino acids, and have been designated rsk-α and rsk-β respectively.The rsk-α cDNA was used to screen murine and avian cDNA libraries. Sequence analysis of full length murine and avian cDNAs show that the enzyme is highly conserved.Recombinant baculoviruses were used to produce recombinant rsk-α and -β protein. Metabolic labelling with ^{35}S-methionine demonstrated a major labelled band in S100 fractions of infected Sf9 cells corresponding to the appropriate molecular mass. Immunoprecipitation of labelled protein using a rabbit antiserum raised against rsk-α antigen produced in E. coli identified a single major band from lysates of cells infected with either the a or b containing recombinant virus. Ribosomal S6 protein kinase assays revealed as much as a 1000-fold increase in activity in insect cells producing the recombinant a protein when co-infected with a v-src recombinant baculovirus. When insect cells were doubly infected by rsk-α and v-src recombinant baculoviruses, a fraction of the rsk-α protein was activated. S-Sepharose chromatography was used to separate active from inactive protein. The active kinase migrated more slowly by SDS-PAGE, increasing its apparent Mr from 82 kDa to 90 kDa. In vitro phosphatase treatment of the active kinase substantially reduced its kinase activity. The baculovirus expression vector system was also used to produce large amounts of inactive rsk-α protein which has been used to study in vitro phosphorylation and subsequent activation of the previously inactive protein. Further studies are underway to determine which kinases are responsible for phosphorylating inactive rsk, and to localize the specific sites where modification of the rsk molecule leads to its activation.

F 323 OVEREXPRESSION OF αPKC LEADS TO A PROLONGED ACCUMULATION OF TPA INDUCED uPA mRNA IN LLC-PK$_1$ CELLS, Markus Wartmann(1), David A. Jans(2), Brian Hemmings(2), Yoshikuni Nagamine(2), Urs Eppenberger(1), Peter J. Parker(3), Doriano Fabbro(1), 1:Laboratory for Molecular Tumorbiology, Department of Research, University Clinic Medical School, Basel, Switzerland, 2:Friedrich Miescher Institute, Basel, Switzerland, 3:Ludwig Institute for Cancer Research, London, UK. Under uninduced conditions the pig kidney cell line LLC-PK$_1$ expresses low levels of urokinase-type plasminogen activator (uPA). Addition of phorbol ester (TPA) results in a transient accumulation of uPA mRNA. In three stably transfected LLC-PK$_1$ cell clones overexpressing α-type PKC (5- to 20-fold), the extent of uPA mRNA induction by TPA is similar to the untransfected cell line. However, the accumulation of the uPA message is significantly prolonged (2-3 fold) in the αPKC overexpressing clones in a TPA dose-dependent manner. This effect coincides with the observation that the TPA induced PKC down regulation is markedly protracted in these clones compared to the parental cell line. This implies that the persistent accumulation of the uPA message observed after induction by TPA is presumably due to the prolonged presence of α-type PKC.

F 324 PURIFICATION AND CHARACTERIZATION OF AN INOSITOL POLYPHOSPHATE-ACTIVATED PROTEIN PHOSPHATASE-PHOSPHORYLATION BY PROTEIN KINASE C, Jean Zwiller[1]and Alton L. Boynton[2]

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Several inositol polyphosphates stimulate the activity of a phosphoprotein phosphatase, Ins (1,3,4,5)P_4 and Ins (1,3,4,5,6)P_5 being the most potent activators. The protein phosphatase originated from a detergent-extract of a particulate bovine brain fraction. The catalytic subunit of this protein phosphatase was purified to homogeneity by sequential chromatography on Heparin-Sepharose, monoQ ion exchange and superose 6 gel filtration. The purified protein phosphatase which was found to be of type-1, exhibits a 36,000 Mr band on SDS-PAGE. The purified enzyme has been shown to be phosphorylated by protein kinase C, but not by protein kinase A or by the Ca^{2+}/calmodulin dependent protein kinase. The phosphorylation of the phosphatase by protein kinase C further enhances the dephosphorylation triggered by the inositol phosphates. The addition of a purified preparation of the protein phosphatase to Ca^{2+}-deprived G1/S blocked T51B rat liver cells stimulates a rapidly responding fraction of cells to enter their S phase and this effect is blocked by protein phosphatase inhibitors heparin and inhibitor-2.

Transcription Factors Induced by Signalling Mechanisms in Growth and Differentiation

F 400 TWO COMMON SITES OF RETROVIRAL INTEGRATION IN AN IL-3-INDEPENDENT MYELOID LEUKEMIA CELL LINE, David S. Askew, Christopher Bartholomew, Art Buchberg, Nancy A. Jenkins, Neal Copeland, and James N. Ihle, Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38105, Mammalian Genetics Laboratory, NCI-Frederick Cancer Research Facility, BRI-Basic Research Program, Frederick, MD 21701.

In order to identify genes that contribute to myeloid leukemogenesis we have cloned viral integration sites from a CasBrM-MuLV induced Il-3 independent myeloblastic leukemia cell line. Genomic probes from flanking cellular sequences were used to screen DNAs from a panel of 30 cell lines derived from CasBrM-MuLV or MoMuLV induced mouse leukemias. Probes from one integration site (Ask-1) defined a region that was rearranged in two additional cell lines, and probes from a second integration site (Ask-2) identified a rearrangement in one additional cell line. Genetic mapping of these loci using interspecific backcrossed mice, assigned the Ask-1 locus to mouse chromosome 2 and the Ask-2 locus to mouse chromosome 19. The Ask-2 site is tightly linked to the Gin-1 locus, a common site of provirus integration in Gross passage A MuLV induced T-cell leukemias. No recombinants were observed between Ask-2 and Gin-1 in 131 mice suggesting that these loci may identify the same region. Interestingly, proviral insertion into the Gin-1 locus has previously been reported only for T-cell malignancies. The Ask-1 locus appears to be a novel site of integration since it maps distinct from any known oncogene or common site of integration of chromosome 2. Northern analysis using a genomic probe derived from the Ask-1 locus identified transcripts ranging in size from 4 kb to 12 kb and was therefore used to screen a cDNA library from the NFS-124 cell line. The characterization of these cDNA clones is in progress.

F 401 LOSS OF FACTOR BINDING TO A FAR UPSTREAM ELEMENT OF C-MYC (FUSE) IS ASSOCIATED WITH CELLULAR DIFFERENTIATION, Mark I. Avigan, Bruce Strober, and David Levens, Laboratory of Pathology, National Cancer Institute, National Institutes of Health 20892

A sensitive exonuclease assay has been employed to analyze binding sites over 2300 bases of c-myc sequence upstream of the P1 promoter. In one set of experiments, protein extracts were prepared from undifferentiated as well as DMSO-treated HL60 and U-937 cells, in order to detect alterations in factor binding concurrent with the shut off of c-myc transcriptional initiation. One factor which binds to a far upstream element (FUSE) approximately 1500 bp 5' of P1, is only present in undifferentiated cells and disappears with commitment to differentiation. The factor has been partially purified by ion exchange chromatography and by oligonucleotide affinity chromatography, and appears to be a 90 kd protein by both Southwestern blot analysis and by UV cross-linking studies. In order to test the fuction of FUSE, a 3.2 kb segment of the c-myc promoter including either FUSE or a mutant with a selective 4 bp deletion within the FUSE binding site, which reduced binding in vitro, was fused to the CAT gene. CAT expression in U-937 transfectants was diminished with the mutant sequence in comparison to the wild-type sequence. However, the deletion had no effect on expression in Hep G2 cells - a line which lacks FUSE binding activity. These results suggest that FUSE has a potentiating effect on c-myc expression when bound by a tissue specific factor which is regulated during differentiation.

F 402 DIFFERENTIAL INDUCTION OF c-jun mRNA ACCUMULATION IN PROMOTION-SENSITIVE AND RESISTANT JB6 CELLS, Elia T. Ben-Ari and Nancy H. Colburn, Cell Biology Section, Laboratory of Viral Carcinogenesis, National Cancer Institute, Frederick, MD 21701.

Clonal variants of mouse epidermal JB6 cells that are susceptible (P^+) or resistant (P^-) to promotion of neoplastic transformation have been used to study the putative role of AP-1/jun in the promotion process. Previous work has shown TPA- and EGF-inducible AP-1 dependent transactivation of gene expression in P^+, but not P^-, variants. The molecular basis for this differential transactivation has now been investigated at the level of jun and fos mRNA accumulation, since the transcription factor AP-1 is composed of heterodimers of jun and fos proteins. Both basal and TPA-inducible c-jun mRNA levels were significantly higher in P^+ than in P^- cells, while there was no difference in EGF-induced c-jun mRNA accumulation in the two cell lines. c-fos mRNA accumulation in response to both TPA and EGF was similar in P^+ and P^- cells. These data suggest that the differential activation of AP-1 function by TPA, but not EGF, in these cell lines is due at least in part to differential regulation at the level of c-jun mRNA accumulation. The induction of jun B and jun D mRNAs in P^+ and P^- cells will also be examined.

F 403 MITOGENIC FUNCTION OF THE MANNOSE-6-PHOSPHATE/INSULIN-LIKE GROWTH FACTOR II RECEPTOR IN NEURONAL PRECURSOR CELLS.

Steen Gammeltoft and Finn C. Nielsen, Department of Clinical Chemistry, Bispebjerg Hospital, DK 2400 Copenhagen NV, Denmark.

The interaction of insulin-like growth factor II (IGF-II) and mannose-6-phosphate (Man-6-P) with their common receptor was studied in primary cultures of neuronal precursor cells from developing rat brain (E15). The Man-6-P/IGF-II receptor bound IGF-II with a Kd of 0.5 nM and insulin-like growth factor I (IGF-I) with 100 times lower affinity. IGF-II bound also to the IGF-I receptor with 10 times lower affinity than IGF-I (Kd ~ 1 nM). Surface-bound [^{125}I]IGF-II was internalized (85% after 60 min) and degraded completely to [^{125}I]tyrosine. Man-6-P increased the the binding affinity of the Man-6-P/IGF-II receptor for IGF-II, without changing the number of binding sites. Neuronal DNA synthesis was increased twofold by IGF-II, but its potency was 10 times lower than IGF-I. Phosphorylated carbohydrates stimulated DNA synthesis twofold compared with mannose. Man-6-P and fructose-1-phosphate were equally potent and glucose-6-phosphate 5 times less potent. The mitogenic effects of Man-6-P, and IGF-II or IGF-I were additive, and antibody 3637 to the Man-6-P/IGF-II receptor blocked the response to Man-6-P but not that to IGF-II or IGF-I. These results show that Man-6-P/IGF-II receptors mediate endocytosis of IGF-II and mitogenesis of Man-6-P in neuronal precursor cells. The cell proliferation induced by IGF-II and IGF-I is mediated by the IGF-I receptor. We conclude that Man-6-P-containing proteins and IGF-II act as mitogens in developing brain by activation of separate receptors.

F 404 THE ACTIVATOR DOMAIN OF TRANSCRIPTION FACTOR CREB IS FUNCTIONALLY AUTONOMOUS. GA Gonzalez and MR Montminy, The Salk Institute, La Jolla, CA 92037

A number of eukaryotic transcription factors appear to be organized into definable transactivating and DNA binding domains. Recent evidence suggests that transactivating regions, in particular, retain function when combined with heterologous DNA binding domains. In the case of transcription factor AP-1, however, DNA binding and dimerization functions appear to be critical for phorbol ester inducible transcription. Here, combinatorial regulation (ie. the formation of jun-fos heterodimers), appear to be the principle mode of control.

CREB is a 341 amino acid nuclear protein whose transcriptional activity is tightly regulated by kinase-A (PKA) mediated phosphorylation at a single serine, SER 133. As substitution of multiple acidic residues for SER 133 does not render the CREB protein constituitively active, negative charge alone is not sufficient to stimulate transcription. The presence of a leucine zipper in CREB which is highly homologous to the corresponding fos and jun motifs prompted us to ask whether, like fos-jun, the formation of CREB heterodimers was critical to transcriptional activation by cAMP. We constructed a chimeric gene containing the putative CREB activator domain upstream of the glucocorticoid receptor DNA binding domain and nuclear localization signal. When expressed from a eukaryotic expression plasmid, this fusion protein, called CREB-GR, confered full cAMP responsiveness to an otherwise unresponsive GRE-CAT reporter construct. Moreover, substitution of the PKA phosphoacceptor serine133 with an alanine rendered CREB-GR completely inactive.

As both CREB-GR and CREB-GR(ALA133) proteins are expressed at comparable levels and are appropriately targeted to the nuclei of transfected cells, these results suggest that the difference in transcriptional activity is attributable to phosphorylation of SER 133 or lack therof. Finally, these results demonstrate that the regulatory effects of cAMP are independent of DNA binding. As CREB is unable to form heterodimer with fos, jun, or myc *in vitro* our data is consistent with the idea that combinatorial regulation is not critical to transactivation by CREB but rather that CREB may act alone to stimulate transcription as a homodimer.

F 405 TUMOR NECROSIS FACTOR (TNF) ACTION IN TA1 CELLS: INDUCTION OF c-fos AND c-jun, Emily M. Haliday and Gordon Ringold, Department of Pharmacology, Stanford University, Stanford, CA 94305-5322

TNF is a 17 kd polypeptide, produced by activated macrophages, that elicits diverse biological effects. Among the more interesting, TNF inhibits the differentiation of adipogenic cells (such as TA1 or 3T3-L1) and when added to mature adipocytes, will cause them to revert to the pre-adipocyte state. We have begun to try to elucidate TNF signal transduction pathways in TA1s with the hope that this will shed light on control of differentiation in these cells. TNF, at 10 ng/ml, induces c-fos and c-jun mRNAs 5-6 fold in quiescent TA1 preadipocytes. c-fos induction is rapid and transient, peaking at 30 minutes, whereas induction of c-jun is maximal at 45 minutes and is more sustained. These responses are useful as markers in studies aimed at identifying the intracellular mediators of TNF action. We are investigating the possibility that arachidonic acid (AA), which is released following TNF treatment, may play such a role. Treatment of TA1 cells with 50uM AA results in an induction of c-fos similar in magnitude and time-course to that with TNF; c-jun, however, is not induced. This suggests that TNF could induce c-fos via AA or one of its metabolites, while TNF induction of c-jun may proceed by a different mechanism. In order to demonstrate that AA induction of c-fos is relevant to TNF-signalling, we are examining the effect of inhibitors of AA metabolism on TNF-fos/jun induction.

F 406 C-FOS PLAYS AN IMPORTANT ROLE IN THE PGE1 MEDIATED INHIBITION OF THE LPS INDUCED TNF RESPONSE IN MACROPHAGES. Alexander G. Haslberger, Claudia Rossbacher, Sandoz Research Institute, and Institute of Genetics and Microbiology, Vienna, Austria. Pretreatment of mouse macrophages for at least 30 minutes with Prostaglandin E1 (PGE1) dose-dependently inhibits TNF release induced by LPS. A similar inhibition of TNF production can be seen in murine macrophages pretreated with sublethal doses of LPS. To investigate a possible role of c-fos in the mechanisms underlying the TNF inhibition in macrophages, c-fos and TNF mRNA accumulation as well as TNF release was analyzed employing Northern blot analysis as well as the TNF-L929 bioassay. In accordance with previous results we found that LPS, like PMA, induces a transient c-fos response with a peak value at 30 minutes decreasing to basal levels at 90 minutes. In the present study PGE1 induces a rapid and strong fos response, lasting between 12 and 16 hrs after a 6 h PGE1 treatment. During the time period of elevated fos levels induced by PGE1, LPS-induced TNF responses were clearly reduced as measured in the bioassay and by mRNA accumulation. Furthermore, addition of LPS alters the kinetics of a pre-existing PGE1 induced fos response. These results suggest an interaction between differential c-fos responses mediated by the different cAMP and PKC pathways. These responses are promoted by the cAMP response element or the dyad symmetry element which can be induced by PMA, EGF, serum and possibly LPS. These interactions may be responsible for the regulation of TNF transcription and thus contribute to PGE1-induced tolerance against endotoxic shock.

F 407 TUMOR CELL ADHERENCE TO CULTURED BOVINE CEREBRAL CORTEX CAPILLARY ENDOTHELIAL CELLS IS PROMOTED BY ACTIVATORS OF PROTEIN KINASE C, J.M. HERBERT and J.P. MAFFRAND, Biochimie Exploratoire, SANOFI RECHERCHE, 195 Route d'Espagne 31036 TOULOUSE FRANCE.

We have evaluated the influence of intracellular Protein kinase C (PKC) activation on the adherence of tumor cells to confluent bovine cerebral cortex capillary endothelial cells (CEC) monolayers *in vitro* . Adherence was assayed with isolated ^{3}H-leucine-labelled L1210. Stimulation of cells with 12-O-tetradecanoyl phorbol 13-acetate (TPA) or phorbol 12,13-dibutyrate (PDBu) increased the rate of adherence of L1210 to CEC. Increased binding was observed with 1 nM of TPA or PDBu with maximal enhancement at 50 nM. 4α-phorbol didecanoate (4α-PDD), known to be inactive for PKC, was without effect in stimulating L1210 adherence to CEC. Pre-incubation studies with TPA (down-regulation experiments) showed that adhesion enhancement was entirely attributable to an effect on tumor cells without contribution of CEC-intracellular PKC. Staurosporine and H7, two protein kinase inhibitors, showed strong antagonist activity on TPA-induced L1210 adherence to CEC (IC50 = 0.5 nM and 10 µM respectively) in relation to their respective inhibitory activities on PKC.
These results strongly suggest that PKC plays a fundamental role in tumor cell adherence to capillary endothelial cells in vitro and that PKC inhibitors may be useful in the prevention of metastasis dissemination.

F 408 THE ROLE OF PROTEIN KINASE A IN *TAX* TRANSACTIVATION OF THE HUMAN T CELL LEUKEMIA VIRUS TYPE 1 LONG TERMINAL REPEAT, Paula Kadison, Harry T. Poteat, Douglas V. Faller, Division of Pediatric Oncology, Dana Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115.

The human T-cell leukemia virus type 1 (HTLV-1) long terminal repeat (LTR) is inducible both by the retroviral *tax* protein and by cyclic AMP in human T cell lines and in murine thymoma S49 cells. Although cyclic AMP transcriptionally activates the LTR in the absence of *tax*, cyclic AMP can also augment the response of the LTR to maximally stimulatory levels of *tax* protein. Therefore, cyclic AMP alone, or in the presence of *tax*, may increase levels of viral structural and regulatory proteins which promote lymphocyte growth. The *cis*-acting sequences that control transcriptional induction by *tax* and by cyclic AMP are in close proximity within the HTLV-1 promotor (LTR). Using a Protein Kinase A (PKA)-deficient S49 mutant cell line, the response of the viral promotor is shown to depend on PKA whereas the response to *tax* does not require the activity of this enzyme. Transactivation of the HTLV-1 LTR by *tax*, however, is decreased in PKA-deficient and adenylate cyclase-deficient cells. The evidence supports largely independent mechanisms of HTLV-1 promotor induction by cyclic AMP and *tax*, but also suggests a role for PKA-mediated phosphorylation in the regulation of HTLV-1 LTR-driven gene expression by *tax* .

F 409 DIFFERENTIATION DEFECTIVE RAS TRANSFECTED MYOBLASTS CAN BE INDUCED TO DIFFERENTIATE BY TIAZOFURIN, David J. Kelvin, T.P.Yamaguchi, J.A.Connolly, S.B. Grove, and William L. Farrar. Biological Carcinogenesis Program, LMI, BRMP, NCI, Frederick, MD. 21701. We have been studying the inhibition of muscle cell differentiation by signal transduction pathways and have previously shown that two separate pathways are involved in the inhibition of BC3H1 differentiation (Kelvin et al. JCB 108:159,168). In an effort to define the role ras plays in these signal transduction pathways we examined the effect tiazofurin, an inhibitor of GTP synthesis, has on Ha-ras transfected muscle cell lines. T-9, a Ha-ras transfected cell line, and parental BC3H1 cells were grown in 20% or 0.5% serum and treated with various concentrations of Tiazofurin for 72 hours. Creatine kinase assays showed that Tiazofurin at 10uM could restore up to 70% of CK activity in 0.5% serum grown T-9 cells when compared to 0.5% serum grown BC3H1 cells. Similar studies done with muscle creatine kinase enhancer CAT constructs showed that Tiazofurin was capable of inducing CAT expression in T-9 cells as well as 20% serum stimulated BC3H1 cells. Preliminary results on the expression of myogenin indicate that Tiazofurin can stimulate expression of this muscle determination gene. The effect of Tiazofurin on T-9 cells indicate that the inhibiting actions of ras are reversible.

F 410 COOPERATIVITY BETWEEN FOS, JUN, AND PROTEIN KINASE C MODULATE THE EGF, BUT NOT THE v-src, INDUCTION OF TRANSIN GENE EXPRESSION. Lawrence D. Kerr & Lynn M. Matrisian. Department of Cell Biology, Vanderbilt University, Nashville, TN 37232

Transin is a transformation-related secreted metalloprotease transcriptionally induced by a variety of growth factors and oncogenes.We have examined the necessity of specific secondary (protein kinase C) and tertiary (c-*fos* and c-*jun* protein products) messengers in the transactivation of transin gene expression by the growth factor, EGF, and the oncogene, v-*src*. Rat-1 fibroblasts stablely transfected with an antisense c-fos construct demonstrated that c-*fos* expression was necessary for the EGF-induction of transin. In a parallel system, Rat-1 fibroblasts transformed with a temperature-sensitive mutant of the Rous sarcoma virus (RSV) demonstrate identical results to those above using c-fos antisense oligonucleotide competetion with EGF at the non-permissive temperature. Sense c-*fos* oligonucleotides had no effect upon the EGF-induction of transin. Similar results demonstrating the necessity of c-*jun* in the EGF-induction of transin have been obtained. In contrast, neither sense nor antisense c-*fos* or c-*jun* oligonucleotides have any effect upon the v-src-induction of transin-promoted CAT activity following a shift to the permissive temperature (35° C). We have also demonsrated the necessity of activated protein kinase C in the EGF-induction of transin CAT activity, mRNA and protein expression. Down modulation of phorbol sensitive C kinase proteins had no effect, however, upon the v-*src* induction of transin. A radiolabelled oligonucleotide corresponding to the GCN4/AP-1 binding site found within the transin promoter specifically bound a protein complex from EGF-stimulated rat fibroblast nuclear extracts but not from control extracts nor v-*src* transformed nuclear extracts. Currently, studies utilizing site-directed mutants of the GCN4/AP-1 binding site within the transin promoter are being employed to examine the effects on EGF and v-*src* induced transin. The data suggest that EGF modulates a C kinase-, c-*fos* /c-*jun*-dependent pathway while v-*src* utilizes a C kinase-, c-*fos* /c-*jun*-independent pathway in the induction of transin gene expression.

F 411 Similar Early Gene Responses to Ligand-Activated EGFR and *neu* Tyrosine Kinases

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The epidermal growth factor receptor (EGFR) and the protein product of the *neu* proto-oncogene are closely related, their tyrosine kinase domains being over 80% homologous. However, their expression is differentially regulated, suggesting distinct roles in cellular development and differentiation. We were interested in comparing the responses of serum-inducible immediate early genes to activation of the epidermal growth factor receptor (EGFR) or a hybrid EGFR/*neu* receptor containing the intracellular domain of the *neu* proto-oncogene. Different receptor-expressing cells were treated with EGF or FCS and analyzed for mRNA expression of 78 genes isolated from serum-stimulated NIH 3T3 cells. About one third of the serum-responsive mRNAs were stimulated by EGFR or *neu* activation. Only few differences in mRNA induction kinetics were found between these tyrosine kinases, emphasizing their functional similarity. In contrast, in cells expressing the constitutively active *neu* oncoprotein the serum-inducibility of the immediate early genes had been lost.

F 412 COORDINATE ACTIVATION OF INTERFERON-STIMULATED GENES REQUIRES CYTOPLASMIC ACTIVATION AND NUCLEAR TRANSLOCATION OF THE HETERODIMERIC TRANSCRIPTION FACTOR ISGF3. David E. Levy, Daniel S. Kessler* and Susan Veals, Department of Pathology, NYU School of Medicine, New York, NY 10016; *Rockefeller University, New York, 10021.

Signal transduction by interferon-α (IFNα) stimulates immediate transcription of a defined set of genes. This coordinate gene activation is dependent upon a conserved cis-acting DNA sequence which is bound by a specific transcription factor, ISGF3. ISGF3 is formed from latent components which pre-exist in cells and are activated following exposure to IFNα. Active ISGF3 initially appears in the cytoplasm of treated cells and is subsequently translocated to the nucleus, a process which can be specifically blocked by treatment with NaF. We have reconstituted *in vitro* one step in the activation of ISGF3. Mixing cytosolic fractions from IFNα treated cells with fractions from untreated cells produces large amounts of active ISGF3. This activation step is non-enzymatic but rather is due to the stoichiometric association of two protein components. One of these components, ISGF3α, is the direct target for activation by IFNα treatment, sustaining a post-translational modification. This modification allows it to associate with the second component, ISGF3γ, and to translocate to the nucleus. The post-translational modification of ISGF3α has been reproduced *in vitro* in permeabilized cells allowing the characterization of the small-molecule requirements and the biochemical nature of this reaction. In addition, we are studying the nuclear translocation of active IGSF3α component *in vitro* using purified protein and isolated nuclei from cells responsive and non-responsive to IFNα. The levels of the latent forms of ISGF3α and ISGF3γ in these cells and the promptness of the nuclear translocation event correlate with the ability of IFNα to inhibit cellular proliferation in normal and IFN-resistant cells.

F 413 2-AMINOPURINE-SENSITIVE PROTEIN PHOSPHORYLATION RESPONSES IN EGF- AND TPA-STIMULATED C-FOS AND C-JUN INDUCTION Louis C Mahadevan, Department of Biochemistry, Oxford University, South Parks Road, Oxford OX1 3QU.

Epidermal Growth Factor (EGF) and Tetradecanoyl Phorbol Acetate (TPA) initiate signalling cascades in C3H 10T1/2 fibroblasts by primarily activating distinct protein kinases, the EGF receptor tyrosine kinase and protein kinase C respectively; there is no signal cross-over at the initiation of signalling. Nevertheless, both agents rapidly elicit common intracellular responses, including the phosphorylation of complexed and chromatin-associated forms of a 33kDa phosphoprotein (pp33) and that of a 15kDa chromatin-associated phosphoprotein (pp15), as well as the transcriptional activation of a common subset of genes including the c-fos proto-oncogene. We show here that 2-aminopurine specifically abolishes complexed and chromatin-associated pp33 phosphorylation in response to EGF and TPA, as well as the induction of c-fos by both agents. The activation of protein kinase C and the levels of transcription factors that bind to the serum response element (SRE), TPA-response element (TRE) or NFkB sites in stimulated cells are unaffected by 2-aminopurine. This, to our knowledge, is the first demonstration that it is possible, by using 2-aminopurine which selectively blocks pp33 phosphorylation, to block c-fos induction in TPA-stimulated cells although protein kinase C remains fully activated. Further, we show here that although TPA-stimulated transcription of c-fos is abolished by 2-aminopurine, the induction of AP-1 is unaffected, suggesting that TPA-stimulated induction of AP-1 utilises existing proteins and is not dependent on fresh C-FOS synthesis. These results imply that 2-aminopurine-sensitive phosphorylation of complexed and chromatin-associated pp33 is crucial to the induction of c-fos, irrespective of the agent used to elicit this response.

F 414 THE NUCLEAR FACTOR CREB IS REQUIRED FOR CYCLIC AMP INDUCED GENE EXPRESSION IN LIVING CELLS, Judy L. Meinkoth, Marc R. Montminy, Stephen I. Fink and James R. Feramisco, Cancer Center, University of California at San Diego, San Diego, CA, The Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, La Jolla, CA and Division of Molecular Medicine, New England Medical Center, Boston MA. Signals emanating at the cell surface are ultimately received in the nucleus and result in altered transcriptional patterns. While the biochemical mediators of the signal transduction process are not entirely known, second messengers and transcriptional activator proteins have been identified in some cases. In many cases the activity of the transcriptional activator proteins is found to be increased following cell stimulation. Although posttranslational modifications have been invoked to explain such increases, the array of molecules responsible for these modifications remain elusive. We constructed mammalian cell lines containing enhancer elements upstream from a readily detectable marker gene. Using an indicator cell line containing cyclic AMP inducible enhancer elements, we show that microinjection of a highly specific antibody to the CREB protein diminishes gene expression in response to cyclic AMP in living cells.

F 415 DOPAMINE RECEPTOR MEDIATED INHIBITORY CONTROL OF C-FOS EXPRESSION IN RAT STRIATUM
Jeannette C. Miller, Department of Psychiatry, Millhauser Laboratories, New York University School of Medicine, New York, New York, 10016
The proto-oncogene, c-fos, encodes a nuclear protein that exhibits DNA binding activity. It has been proposed to be involved in cell proliferation and differentiation. There are a number of reports showing that c-fos mRNA levels are increased in rat brain by a variety of drugs, including agents that provoke voltage-dependent changes in calcium influx, Ach receptor stimulation, and seizures. It has been proposed that c-fos may function as an initial step in coupling short-term signals via activation of cell membrane receptors to long-term adaptive responses in the cell. One such possibility may include alterations in receptor number known to occur in neuronal cells after neuropharmacological intervention. The dopaminergic system in brain is known to respond in an adaptive manner to neuroleptic drug exposure by increasing the number of DA, D2 receptors. In the present studies, the expression of c-fos mRNA in rat striatum was investigated by Northern hybridization to a 1.0 kb fragment of c-fos cDNA after exposure to the DA, D2 antagonist and neuroleptic, haloperidol. The expression of c-fos mRNA was found to be rapidly and transiently induced by exposure to haloperidol, when compared to the expression in animals receiving saline. The c-fos response was also found to be dose-dependent. Studies using selective DA, D1 and D2 receptor agents showed that this induction of c-fos expression by neuroleptic is D2 receptor mediated. These findings reveal that c-fos expression in striatum is under DA, D2 inhibitory control and suggest that c-fos may play an important role in the initiation of neuroleptic-induced alterations in D2 receptor number.

F 416 FOS PROTEIN: PHOSPHORYLATION OF C-TERMINAL IS OBLIGATORY FOR TRANS-SUPPRESSION BUT NOT FOR TRANSACTIVATION, Rivka Ofir and Inder M. Verma, Molecular Biology & Virology Laboratory, The Salk Institute, La Jolla, CA 92037
The product of fos proto-oncogene is involved in regulation of transcription of several genes either by transactivation or by transrepression. Induction of transcription of fos gene is transient and requires conserved 5' regulatory elements. Down regulation of c-fos transcription that occurs following serum-induced activation is likely due to autoregulation of fos promoter by fos protein. Mutation at the C-terminus of fos protein, of the type found in a viral fos protein prevent down regulation of c-fos transcription following serum induction. Viral and cellular fos protein differ in their carboxyl termini and it has been shown that phosphorylation of c-fos protein, but not v-fos protein can be stimulated in vivo by the addition of either TPA or serum. It is very likely that phosphorylation will have a profound effect on the biological properties of the protein. The c-fos unique sites of phosphorylation reside in the C-terminal 20 amino acids. This sequence is missing in the v-fos gene. To determine the effect of phosphorylation on the functional properties of the c-fos protein we mutagenized the different serine residues in the C-terminal of c-fos protein. The resulting proteins were tested for transrepression as well as for transactivation by transient transfection assay. The results indicate that mutations of serine residues in the C-terminal result in a protein which is unable to transrepress. In contrast the ability of these "mutated" fos proteins to transactivate TRE-linked transcription is not compromised. These results show that transrepression and transactivation domains in the fos protein can be delineated.

F 417 **PHOSPHORYLATION OF DNA TOPOISOMERASE I AND REGULATION BY PROTEIN KINASE C.** Yves Pommier*, Donna Kerrigan*, and Robert I. Glazer#. *Laboratory of Molecular Pharmacology and #Laboratory of Biological Chemistry, DTP, DCT, NCI.
DNA topoisomerases I (topo I) are nuclear enzymes which relax supercoiling and relieve torsional tension in DNA. Since DNA replication and gene transcription are likely to induce torsional tension, it appears critical to determine the cellular factors regulating topo I activity. Also topo I is the cellular target of camptothecin, an antitumor agent whose derivatives are currently investigated as anticancer agents. Camptothecin stabilizes topo I-DNA complexes in which the enzyme is covalently linked to the 3'-DNA termini of a DNA single-strand breaks made by the enzyme. In the present study, we have investigated the influence of the phosphorylation of topo I on enzyme activity. Dephosphorylation of topo I by calf intestine alkaline phosphatase (CIAP) abolished both DNA relaxation activity and induction of DNA breaks by camptothecin. Topo I could be reactivated by incubating CIAP-inactivated enzyme with purified protein kinase C. Reactivated topo I was able to relax supercoiled DNA processively, like the native enzyme, and cleaved ^{32}P-end-labeled DNA fragments at the same sites as the native enzyme. These results show that active topo I is phosphorylated and suggest that phosphorylation by kinase C may regulate topo I catalytic activity and sensitivity to camptothecin.

F 418 A Functional Role for the Catalytic Subunit of Protein Kinase A in HTLV-1 Gene Expression. H.T. Poteat, M. Dardik, P. Kadison, U. Ramstedt, A. Hey, J.G. Sodroski, and W.A. Haseltine, Division of Human Retrovirology, Department of Pathology, Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA 02115

The long terminal repeat sequence (LTR) of the Human T cell Leukemia virus type one (HTLV-1) contains an imperfect repeat of 21 nucleotides which governs the response to the virus trans-activator protein tax and to cyclic AMP. In a murine thymocyte cell line defective in the catalytic subunit of protein kinase A, the response of the HTLV-1 LTR to cyclic AMP is abolished and the response to tax is substantially diminished. This report shows that a factor present in nuclear extracts of wild type cells binds to the HTLV-1 21 nucleotide sequence and that this binding activity is missing from the extracts of protein kinase A defective cells. Treatment of nuclear extracts of protein kinase A defective cells with bovine protein kinase A catalytic subunit restores the binding activity, whereas treatment of wild type nuclear extracts with a protein phosphatase destroys the binding activity. Gamma-S-ATP when present during kinase treatment of the deficient extracts, inhibits complex formation. In a related set of experiments the catalytic subunit of bovine protein kinase A was introduced into Hela cells using the technique of scrape loading. Hela cells transfected with a plasmid construct containing the acetyl-transferase gene downstream of the HTLV-1 LTR showed induction of marker gene expression when the catalytic subunit was introduced via scrape loading. These results indicate that in murine thymocytes the response of the HTLV-1 LTR to cyclic AMP depends, at least in part, upon the binding of a phosphorylated protein to the 21 nucleotide repeat sequence and that the response to tax is partially dependent upon binding of the phosphorylated protein. The results suggest a model in which the phosphorylation of transcriptional factors regulates HTLV-1 gene expression.

F 419 CELL CYCLE-SPECIFIC ACTION OF NERVE GROWTH FACTOR IN PC12 CELLS: DIFFERENTIATION WITHOUT PROLIFERATION, **Brian B. Rudkin**, Philip Lazarovici, Ben-Zion Levi[1], Yuya Abe[2], Ko Fujita and Gordon Guroff, **Section on Growth Factors** and [1]Laboratory of Developmental and Molecular Immunity, Section on Molecular Genetics of Immunity, **National Institute of Child Health and Human Development**, [2]Laboratory of Biological Chemistry, Division of Cancer Treatment, Developmental Therapeutics Program, National Cancer Institute, **National Institutes of Health, Bethesda, MD 20892**. PC12 cells were manipulated in such a way as to permit the study of differentiation-specific responses independently from proliferative responses. Cells were starved for serum then exposed to nerve growth factor (NGF) or serum. Following addition of serum, cells incorporated thymidine in a synchronous manner. Subsequent to the wave of DNA synthesis, the cell number increased approximately two-fold. Addition of NGF to serum-starved cultures had no measureable effect on either parameter. Neurite outgrowth was more rapid and extensive, and appearance of Na^+ channels, measured as saxitoxin binding sites, more rapid than when NGF was added to exponentially-growing cells. Epidermal growth factor receptors were heterologously downregulated by NGF with similar kinetics under both conditions. Induction of the proto-oncogene c-fos by NGF was also greater in the serum-starved cells than in exponentially-growing cultures. These results indicated that serum starvation resulted in synchronisation of the cultures and that NGF action may be cell cycle-specific. Analysis of the cellular response to NGF at different times during the cell cycle showed that c-fos was induced in the G_1 phase but not in S or G_2. Fluorescence-activated cell sorter analysis demonstrated that addition of NGF to exponentially-growing cells, resulted in their accumulation in a G_1-like state. With regard to the study of the mechanism of NGF action, these results illustrate that measurements of NGF effects on specific components in the signal transduction pathway may be confounded by the use of exponentially-growing cultures.

F 420 COMPARATIVE STUDIES ON THE BINDING OF JUN PROTEINS TO AP-1- OR CRE-CONTAINING OLIGONUCLEOTIDES: EFFECT OF FOS PROTEINS. Rolf-Peter Ryseck and Rodrigo Bravo, Department of Molecular Biology, The Squibb Institute for Medical Research, Princeton, NJ 08543.

Using gel retardation assay the *in vitro* binding activities of JUN, JUN B and JUN D proteins alone or in complexes with FOS, FOS B and FRA-1 proteins have been determined. For these studies, different oligonucleotides containing the AP-1 (TGACTCA) or CRE (TGACGTCA) consensus sequences were used. The results obtained demonstrate that JUN protein binds with different affinities to different oligonucleotides which contain an identical AP-1 binding site, implying that the adjacent sequences play a role in stabilizing the JUN-DNA complex. Interestingly, an AP-1 containing oligonucleotide which binds JUN with high affinity can be transformed to a CRE-containing oligonucleotide which will also bind JUN very efficiently. Accordingly, a low affinity AP-1 oligonucleotide will only generate a low affinity CRE-oligonucleotide. However, JUN complexed with either FOS, FOS B or FRA-1 proteins binds to all AP-1- and CRE-containing oligonucleotides with similar affinities.

Comparison of the binding properties of JUN, JUN B and JUN D proteins reveals that the order of their affinities for different AP-1- or CRE-containing sequences is JUN > JUN D > JUN B. However, in complex with either FOS, FOS B or FRA-1 proteins each of the three JUN proteins binds with similar affinities to the different AP-1- and CRE-containing oligonucleotides.

F 421 Downregulation of the Early Genomic Growth Factor Response in *neu* and c-Ha-*ras* Oncogene-Transformed Cells

Lea Sistonen, Päivi Koskinen, Heikki Lehväslaiho, Laura Lehtola, Rodrigo Bravo* and Kari Alitalo, Departments of Virology and Pathology, University of Helsinki, 00290 Helsinki, Finland; *The Squibb Institute for Medical Research, Department of Molecular Biology, Princeton, NJ 08543-4000, USA.

Signal transduction leads to long-term adjustment of cellular genetic programs to biochemical signals mediated by polypeptide growth factor receptors. A point mutation in the transmembrane domain of the *neu* receptor has been found to activate its tyrosine kinase activity and oncogenic potential. Molecular modelling suggests that the effect of the mutation is enhanced dimerization of the *neu* oncoprotein which may mimick the dimerization of growth factor receptors induced by their ligands (1). We have shown that ligand stimulation of a chimeric epidermal growth factor receptor-*neu* proto-oncogene induces its tyrosine kinase, an increased membrane potential, intracellular calcium, a programmed activation of cell growth-regulated genes and DNA synthesis (2-5). The *neu* and c-Ha-*ras* oncoproteins inhibited quiescence upon serum starvation and prevented 50-90% of the serum-stimulated mRNA induction of most immediate early serum response genes, such as *jun*B encoding a transcription factor, N10 encoding a putative nuclear hormone binding receptor for an as yet undefined ligand. The responses to EGF and the tumor promoter TPA were inhibited by 80-95% and 70-80%, respectively. Thus the *neu* and c-Ha-*ras* oncogenes deregulate the genetic response programs for extracellular signalling.

1. Sternberg and Gullick, Nature 339: 587, 1989.; 2. Lehväslaiho et al., EMBO J. 8: 159-166, 1989.; 3. Lehtola et al., Growth Factors, in press.; 4. Sistonen et al., J. Cell Biol., in press.; 5. Pandiela et al., Oncogene, in press.

F 422 HORMONE-DEPENDENT ACTIVITY OF FOS-STEREOID RECEPTOR FUSION PROTEINS, Giulio Superti-Furga *, Didier Picard ° and Meinrad Busslinger *, Institute for Molecular Pathology ,Vienna, Austria (*) and Department of Biochemistry and Biophysics,UCSF, San Francisco (°) The hormone-binding domain of the estrogen and glucocorticoid receptors contains an inactivation function that renders the activity of a protein fused to it ligand-dependent. The trans-activation function of the adenovirus E1A protein and the transforming activity of the Myc protein have been successfully put under hormonal control this way (Picard et al. Cell 54, 1073, Eilers et al. Nature 340, 66). We have constructed chimaeras between the mouse c-Fos or v-Fos and the C-terminal region of the human estrogen or the rat glucocorticoid receptor. In transient transfection experiments these hybrid proteins are able to trans-activate a test gene containing AP1 binding sites in a hormone-dependent manner in NIH3T3, HeLa, CV-1 and F9 cells. We are currently investigating the hormone-dependent transformation potential of these molecules by stable integration of the genes in NIH3T3 and 208F cells. It will be interesting to see whether these hybrid proteins are the equivalent of conditional Fos mutants that will allow careful timing and titration of Fos activity and that will therefore be a useful tool to study the role of Fos in transformation.

F 423 T CELL ACTIVATION SIGNALS REGULATE HIV GENE EXPRESSION, Sandra Tong, Terry Welsh, and B. Matija Peterlin, Howard Hughes Medical Institute, University of Californis, San Francisco, CA 94143

Signals for activation and proliferation of T cells also account for conversion from latent human immunodeficiency virus (HIV) infection to productive infection in T cells. We have compared regulation of HIV1 and 2 gene expression in activated T cells. With the use of transient expression assays in the human CD4+ T cell line, Jurkat, HIV1 long terminal repeat (LTR) is more responsive to phorbol esters (PMA) and phytohemagglutinin (PHA) than HIV2 LTR. When placed upstream from a heterologous promoter, HIV1 enhancer is sufficient for the response to PMA and PHA but does not function in the uninduced state. HIV1 enhancer consists of two binding sites for nuclear factor κB (NFκB), a second messenger required for expression of interleukin-2 and its receptor in activated T cells. In contrast, HIV2 enhancer contains one conserved and one divergent NFκB binding site. When placed upstream from a heterologous promoter, HIV2 enhancer displays basal enhancer function and is less responsive than HIV1 enhancer to T cell activation signals; the lack of constitutive enhancer function in the context of the wild-type LTR may be due to the HIV-2 trans-acting responsive region acting as a negative element. Placement of HIV2 enhancer in HIV1 LTR results in higher basal expression and a decrease in the response to T cell activation signals. Conversely, when two intact NFκB sites are placed in HIV2 LTR, we observe an increased response to PMA and PHA, but the effect is not synergistic. Protein gel retardation assays show that the two NFκB sites are not bound simultaneously, which suggests that steric restrictions may limit cooperative function of two sites. In addition, the divergent site in HIV2 enhancer does not bind NFκB but does interact with a different set of inducible protein complexes. Our findings may explain the longer period of latency seen in HIV2 infections before the clinical onset of AIDS.

F 424 MECHANISM OF MUSCARINIC RECEPTOR-MEDIATED PROTO-ONCOGENE EXPRESSION IN 1321N1 ASTROCYTOMA CELLS. J. Trejo and J. H. Brown, Dept. of Pharmacology, U. C. San Diego, La Jolla, CA 92093

The muscarinic cholinergic receptors (mAChR) expressed in 1321N1 astrocytoma cells are coupled to the phosphatidylinositol signal transduction pathway leading to Ca^{2+} mobilization and protein kinase C (PKC) activation. Receptor-stimulated increases in cytosolic [Ca^{2+}], activation of PKC, and increases in intracellular levels of cAMP have been linked to the regulation of the proto-oncogene c-fos. To determine whether activation of the mAChR leads to changes in the expression of c-fos and other proto-oncogenes, the levels of mRNA transcripts were measured by Northern analysis. Stimulation of the mAChR by carbachol in quiescent 1321N1 cells resulted in a 20-fold increase in c-fos mRNA. This increase in c-fos expression was detected as early as 15 minutes, peaked at 30 minutes, and was no longer detectable by 2 hours. The response to carbachol was blocked by the mAChR antagonist atropine. Carbachol also led to the increased expression of c-jun with a time course of induction similar to that of c-fos. In contrast to the transient induction of c-fos and c-jun, the expression of c-myc induced by carbachol peaked at 1 hour and remained elevated for at least 4 hours. To determine the role of PKC in mAChR stimulated c-fos induction we down-regulated PKC by exposing the cells to 1 μM PMA for 16 hours. Under these conditions, PKC immunoreactivity was lost and mAChR-mediated c-fos induction was virtually abolished. These data implicate PKC in the sequence of events leading to c-fos induction. However, PKC activation by phorbol ester alone increased c-fos expression to a lesser extent than carbachol, suggesting that other mAChR-mediated signals such as Ca^{2+} mobilization may contribute to the expression of c-fos. To assess the importance of Ca^{2+} mobilization resulting from receptor activation, the Ca^{2+} transient was buffered with quin-2 or BAPTA. This manipulation attenuated the mAChR-mediated increase in c-fos mRNA. The mAChR-mediated increase in cytosolic Ca^{2+} may enhance the induction of c-fos by acting through an indirect mechanism involving PKC. Evidence supporting this hypothesis is that the increase in c-fos mRNA induced by 1 μM ionomycin was significantly reduced in PKC-down-regulated cells. We conclude that mAChR stimulation increases c-fos mRNA through a mechanism dependent on PKC and supported by a rise intracellular Ca^{2+}.

F 425 PLATELET-DERIVED GROWTH FACTOR INDUCES SPECIFIC GENE EXPRESSION AND INITIATION OF DNA SYNTHESIS DEPENDENT ON INCREASED FREE CYTOSOLIC CALCIUM, Robert W. Tucker, Danielle Ferris, John 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 may instead involve the direct activation of gene expression by intracellular signals. We have previously reported that PDGF-stimulated DNA synthesis in quiescent BALB/c 3T3 cells requires increases in free cytosolic calcium (Ca_i), even though Ca_i increases induced by calcium ionophore A23187 are not sufficient to induce DNA synthesis. Here we describe experiments on the relationship between Ca_i increases and gene expression stimulated by PDGF. We used digital image analysis of Fura 2-loaded 3T3 cells to measure Ca_i transients, and in-situ hybridization with antisense ^{35}S-RNA probes to detect increased accumulation of specific mRNA's encoded by c-fos, c-jun, jun-b and 268 genes. As previously reported, mitogenic concentrations (30 ng/ml) of PDGF (BB homodimer) increased mRNA levels of all these immediate early genes. When Quin 2/AM was used to buffer intracellular Ca_i transients induced by PDGF, only 268 gene expression was still induced by PDGF; mRNA concentrations of c-fos, c-jun, and jun-b all failed to increase in Quin 2-loaded cells stimulated with PDGF. Ca_i increases induced by calcium ionophore did not appear to be sufficient to induce increased expression of any of these immediate early genes. Since fos and jun proteins form dimers that bind to specific sites on DNA and initiate new gene transcription, it is particularly interesting that PDGF induces c-fos and jun-b mRNA accumulation via a common intracellular signal (Ca_i increases), while some other genes (e.g. 268) are independent of Ca_i increases induced by PDGF.

F 426 **OCT-1 OR A SIMILAR PROTEIN TRANSMITS A CA^{++} INDUCED SIGNAL INVOLVED IN T CELL ACTIVATION** Katharine S. Ullman*, Cynthia A. Edwards+, and Gerald R. Crabtree*. *Howard Hughes Medical Institute, Beckman Center. Stanford University. Stanford, CA, 94305. +Genelabs, Inc. 505 Penobscot Drive. Redwood City, CA. 94063

Interleukin-2 (IL-2) is one of the genes which becomes transcriptionally active during the early stages of T lymphocyte stimulation. We have characterized IL-2 enhancer binding proteins in order to understand how their activity is regulated and ultimately what links these proteins to the initial signals at the membrane. NF-IL2-A binds to sequences from -63 to -93 in the IL-2 enhancer and is essential for IL-2 enhancer activity. Tandem repeats of the NF-IL2-A site confer inducible transcription from a heterologous promoter in the T lymphoma line Jurkat following signals that mimic the physiological events of T cells activation such as the combination of phorbol esters and either antibody against the T cell receptor or a calcium ionophore. Recently we have found that NF-IL2-A is indistinguishable from Oct-1, a multi-functional homeo-domain containing protein. NF-IL2-A was purified and found to have the same molecular weight as Oct-1. The DNA-protein complexes formed with sequences corresponding to the binding sites for NF-IL2-A and Oct-1 comigrate in gel mobility shift assays and have similar DNA sequence specificity. In addition, partial protease digestion of these complexes results in indistinguishable patterns. We have also found that an Oct-1-β-galactosidase fusion protein produced in bacteria binds specifically to the NF-IL2-A binding site. These results indicate that Oct-1, or a closely related protein, acquires transcriptional activity in response to a calcium signal transmitted to the nucleus following T cell activation. A calcium responsive role of Oct-1 in other cell types has not yet been explored.

F 427 CHARACTERIZATION AND CLONING OF TRANSCRIPTION FACTORS INVOLVED IN CELL SIGNALLING BY IFN AND PDGF. Bryan R.G. Williams, Greg Hannigan, Aseem Kumar, S. Jaharul Haque and Michael Rutherford, Research Institute, Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8.
In a number of interferon (IFN) responsive genes a highly conserved consensus sequence (ISRE) of 13 or 14 bp confers IFN inducibility. We have shown that the ISRE of the 2-5A synthetase gene also confers responsiveness to PDGF. The signalling of the ISRE by IFN and PDGF appears to be mediated through distinct signal transduction pathways. The PDGF response is sensitive to inhibition by the PKC inhibitor, staurosporine whereas the IFN response is not affected by this inhibitor. Both IFN and PDGF signal the binding of similar proteins to the ISRE. UV-crosslinking experiments indicate a constitutive protein complex as well as an induced complex which appears within minutes of addition of either inducer. The induced complex appears to consist of a dimer of 65 kd proteins. In order to characterize further, transcription factors involved in IFN signalling we have screened cDNA expression libraries with probes containing the ISRE. Three proteins have been cloned which closely resemble those detected by UV-crosslinking. Functional characterization of these proteins and analysis of the mechanism of signalling indicates activation of a novel signalling pathway by IFN.

F 428 CHARACTERIZATION OF AN ALTERNATELY SPLICED TRANSACTIVATING DOMAIN IN CREB, Karen K. Yamamoto, G.A. Gonzalez, P. Menzel and M.R.Montminy, Salk Institute, La Jolla, CA 92037
The structural features of trans-activating domains for a number of transcription factors have recently been described. Among these, amphipathic helices figure prominently because of their potential to promote protein-protein interactions. CREB is a eukaryotic transcription factor which induces transcription of a number of eukaryotic genes in response to cAMP. We have recently isolated a cDNA clone for CREB which predicts a protein of 341 amino acids. We have also obtained a CREB-related cDNA, named ΔCREB, which contains a 14 amino acid deletion from residues 88 to 101. CREB and ΔCREB cDNAs are identical, except for this deletion, and appear to represent alternately spliced products from a single gene. To determine whether CREB and ΔCREB could be distinguished functionally, we performed a series of transient transfection assays using eukaryotic expression vectors encoding each form. We observed that CREB had 10-fold higher activity than ΔCREB, indicating that this 14 amino acid region, termed α peptide, is necessary for maximal cAMP-dependent induction. To investigate the basis for the differences in activity between CREB and ΔCREB proteins, the secondary structure of the α region was examined. Circular dichroism analyses of a synthetic α peptides reveals a spectrum indicative of α helical structure. Furthermore, a helical wheel diagram of residues 89-96 suggests that this peptide may form an amphipathic α helix containing hydrophobic residues on one face, and mainly basic residues on the other. To determine the importance of secondary structure within the α peptide to the transactivation potential of CREB, we designed several single amino acid substitution mutants. Cotransfection studies with these mutants show that a helix disrupting (Leu to Pro) substitution within the α peptide renders this mutant almost completely inactive, whereas a conservative Cys to Ser mutant, within the same region, has intermediate activity. Our results demonstrate that the α peptide of CREB is critical to the transactivation potential of this protein and that the secondary structure of this peptide is particualrly important for this effect.

Hormone Receptor Signalling

F 600 **INTERMOLECULAR TRANSPHOSPHORYLATION BETWEEN INSULIN RECEPTORS AND EGF-INSULIN RECEPTOR CHIMERAE,** **Robert Ballotti, Reiner Lammers[2], Jean-Claude Scimeca, Thomas Dull[2], Joseph Schlessinger[1], Axel Ullrich[2], and Emmanuel Van Obberghen,** INSERM U 145, Nice, Cédex 06034, France; Depart. Pharmacology[1], New York University , New York and Max-Planck Institut für Biochemie[2], München, FRG.
The insulin receptor, a glycoprotein consisting of two extracellular α– and two transmembrane ß-subunits, is thought to mediate hormone action by means of its tyrosine-specific protein kinase activity. To explore the mechanism of insulin receptor phosphorylation we have used NIH 3T3 cells transfected with two receptor constructs: one encoding a chimeric receptor composed of the extracellular domain of the human EGF receptor and the cytosolic domain of the human insulin receptor ß subunit, and a second construct encoding a kinase-deficient human insulin receptor.Stimulation of these cells with EGF induced tyrosine autophosphorylation of the EGF-insulin receptor chimera (150 kd) and tyrosine phosphorylation of the ß subunit of the kinase-deficient insulin receptor (95 kd). The phosphopeptides of the autophosphorylated cytoplasmic domain of the EGF-insulin receptor chimera were comparable to those of the transphosphorylated ß subunit of the kinase-deficient insulin receptor and of the wild type human insulin receptor. When immunoaffinity purified EGF-insulin receptor hybrids and kinase-deficient insulin receptors were used in a cell lysate phosphorylation assay, it was found that addition of EGF produced [^{32}P]– labeling of both receptor species. We conclude that EGF acting directly through the EGF-insulin receptor chimera causes transphosphorylation of the kinase-deficient insulin receptor. These data support the notion that autophosphorylation of the insulin receptor may proceed by an intermolecular mechanism.

F 601 ENHANCED CELL PROLIFERATION IN HYPERTENSION:DISSOCIATION BETWEEN INOSITOL PHOSPHATES AND DIACYLGLYCEROL PRODUCTIONS. Maryvonne Baudouin- Legros, Jean-Luc Paquet, Philippe Meyer. INSERM U7, Hopital Necker, 75015 PARIS FRANCE.
Aortic smooth muscle cells from spontaneously hypertensive rats (SHR) proliferate more actively than control cells (WKY) under FCS stimulation. Serum-induced inositol phosphates production is equivalent in both types of cells (600% of basal production) and so are c-jun and c-fos expressions. However, in the same time, arachidonate-diacylglycerol is significatively higher in SHR cells than in controls. On the other hand angiotensin II (a weak mitogenic agent on these cells) is more active on SHR cells than on WKY ones concerning 3H thymidine incorporation, both IPs and DAG formations and the proto-oncogene expressions. The highest maximal levels of both IPs and DAG are not reached in the presence of serum but under ang.II stimulation. Enhanced SHR cells mitogenic response to FCS therefore cannot be explained either by increased phosphoinositide turn-over or by another phospholipid hydrolysis which is however stimulated by both FCS and angiotensin in aortic smooth muscle cells.

F 602 TYROSINE PHOSPHORYLATION POSITIVELY REGULATES THE TRANSFORMING ABILITY OF THE HUMAN EGF RECEPTOR. L.Beguinot, K. Helin, T.J. Velu, D.R. Lowy. Microbiology Institute, University of Copenhagen,DK-1353 Denmark, Laboratory of Cellular Oncology, NIH, Bethesda MD 20892 USA.
The full lenght EGF receptor is able to transform cells in culture in the presence of EGF and has been implicated in human tumors.In contrast to v-erbB, deletion of its last 63 aa (Dc63) drastically decrease its transforming potential.The EGF receptor C-terminus contains three conserved tyr (Y-1068, Y-1148,Y-1173) which become phosphorylated when it is activated by EGF.To clarify the role of the two last Tyr deleted in Dc63 and the function of tyr 1068, each Tyr has been mutated to Phe and studied as single, double and triple mutant in the full lenght receptor.While the EGF-dependent transforming activity of the single point mutants is similar to that of the wild type, that of the double mutants is decreased 3 to 5 fold, an even lower activity is present in the triple mutant.EGF-dependent foci, growth in agar and growth in low serum are similarly impaired.Autophosphorylation is decreased in the double and triple point mutants and a lower response is observed in some of the EGF-dependent early events (as PIP hydrolisis and raise in cytoplasmic Ca) indicating a decrease in phosphorylation of endogeneous substrates.We conclude that the C-terminus of the EGF receptor positively regulate its biological activity via tyr autophosphorylation.These results may provide an explanation for the failure to find tumors with EGFR with alterations in the C-terminus.

F 603 DIFFERENTIAL REGULATION OF THE Na^+/H^+ EXCHANGER BY HYPERTROPHIC AND HYPERPLASTIC GROWTH STIMULI IN VASCULAR SMOOTH MUSCLE. Bradford C. Berk and Masayuki Mitsuka, Division of Cardiology, Dept of Medicine, Emory University School of Medicine, Atlanta, GA 30322.
Mitogens such as serum and platelet-derived growth factor (PDGF) stimulate a rapid (< 1 min) rise in intracellular pH (pH_i), mediated by Na^+/H^+ exchange (NaH), in cultured rat aortic smooth muscle cells (RASM). We have shown that the vasoconstrictor angiotensin II (AII) stimulates NaH and causes a hypertrophic growth response, characterized by increases in cell protein, cell volume and ^3H-leucine incorporation, but not cell number. Because of the importance of NaH in growth, we hypothesized that long term regulation of NaH might differ in hypertrophic vs hyperplastic RASM. To measure changes in NaH function, RASM were growth arrested for 48 hr in 0.4% serum and then exposed for 24 hr to mitogenic (10 ng/ml PDGF, 10% serum) or hypertrophic (100 nM AII) agonists. Changes in NaH function appeared likely because significant changes in resting pH_i were observed: 7.11 (0.4% serum), 7.18 (10% serum, PDGF) and 6.95 (AII). NaH was measured by the ethylisopropyl amiloride (EIPA)-sensitive influx of Li^+, (normalized to cell protein by simultaneous assay of K^+). Total Li^+/K^+ influx decreased from 3.12 $\pm$ 0.23 (0.4% serum) to 1.10 $\pm$ 0.18 (10% serum), with a 63% decrease in EIPA-sensitive influx from 2.46 to 0.90. Similar decreases were observed following PDGF (43%), but not AII (5%). This decrease appeared to be mediated by protein kinase C (PKC) because treatment with 200 nM phorbol 12,13 dibutyrate caused a 63% decrease in EIPA-sensitive influx, but the inactive 4α-phorbol didecanoate caused no significant change. These results suggest that regulation of NaH may be mediated by changes in pH_i or PKC signal transduction. Altered regulation of the Na^+/H^+ exchanger is one of the first reported differences between hyperplastic and hypertrophied cells.

F 604 RETINOIC ACID REGULATES KERATIN GENE EXPRESSION VIA ITS NUCLEAR RECEPTORS, M. Blumenberg, M. Tomic, H.S. Epstein, C.-K. Jiang, I.M. Freedberg, M. Petkovich*, and P. Chambon*, Department of Dermatology, NYU Medical Center and *Institute de Chimie Biologique,, Faculte de Medecine, Strasbourg, France.

We have linked the promoter region of a human K#14 keratin gene to the chloramphencol acetyl transferase reporter gene in order to elucidate the elements required for regulation of keratin expression in the epidermis. This contruct was introduced into mammalian cell lines and primary cultures via calcium phosphate-mediated DNA precipitation. By co-transfecting with constructs expressing the retinoic acid receptor gene we have shown that retinoic acid receptor regulates keratin gene expression in ligand dependent manner. However, unlike other genes regulated by the retinoic acid receptor, keratin genes are turned off by the action of the nuclear receptor when bound to retinoic acid. The effect of retinoic acid and its receptor was evident both in transformed epithelial cell lines such as HeLa and in primary cultures of keratinocytes. Our results indicate that retinoic acid directly regulates keratin gene expression via its nuclear receptor.

F 605 IDENTIFICATION OF A TGF-β AND cAMP INDUCIBLE REGULATORY REGION IN THE HUMAN ELASTIN PROMOTER, Giorgio M. Bressan and Keith K. Stanley, Institute of Histology University of Padova, 35100 Italy and , EMBL, 6900 Heidelberg, FRG

Several genes for components of the extracellular matrix and their receptors are under the control of different growth factors. TGF-β is one of the most powerful regulators of the expression of these genes, having usually a stimulating effect. It has recently been reported that elastin, the major component of elastic fibers, is also a target for TGF-β action in fibroblasts. As a first step in defining the molecular mechanism of transcriptional activation of the elastin gene by TGF-β, we performed transient DNA transfer experiments with elastin-chloramophenicol acetyl-transferase fusions. Two functionally different segments were identified in the 5' flanking region of the human elastin gene. Sequences 200 bases upstream from the start site of translation (elastin has no TATA box and transcription starts at several sites), increased expression similarly in induced and uninduced cells, while the region from -17 to -196 was responsible for induction by TGF-β (more than 10 fold) and drugs increasing intracellular cAMP (4-5 fold). Deletion of this region abolished expression of the constructs. This segment contains the consensus sequence for an AP2 binding site and a sequence homologous to the core motif recognized by CREB proteins. Two sequences which partially overlapped with these binding sites were protected in footprint experiments.

F 606 HORMONE-INDUCED HYDROLYSIS OF CELLULAR POLYPHOSPHOINOSITIDES BY PHOSPHOLIPASE C AND PHOSPHATIDYLCHOLINE BY PHOSPHOLIPASE D: *A TIME SEQUENCE STUDY OF THE GENERATION OF SECOND MESSENGERS*, Myles C. Cabot and Chunfa Huang, W. Alton Jones Cell Science Center, Lake Placid, NY 12946

Studies on hormone-induced activation of cellular phospholipase D (PLD) have raised several interesting questions concerning the roles of nonphosphoinositide-derived phosphatidate (PA), a purported modifier of cell response, and diacylglycerol (DG). In order to address these questions, examination of the possible coordination of phospholipid degradative pathways and their temporal relationship must be ascertained. In REF52 rat fibroblasts, prelabeled with [^{3}H]arachidonic acid, vasopressin (VP) treatment induced phosphatidylinositol 4,5-bisphosphate (PIP_2) hydrolysis by PLC (measured by [^{3}H]DG formation) at 5-15 sec. At 15 sec the induction of PLD activity, measured by the generation of PC-derived PA, ensued. With ethanol in the culture medium, VP elicited the formation of PC-derived phosphatidylethanol, (catalyzed by PLD transphosphatidylation), which occurred with a time frame that paralleled the formation of PA in the absence of ethanol. PA generation from PC was maximal at 2-2.5 min, at which time the formation of PC-derived DG was initiated. Hormone induced PLD activity was also demonstrated in vascular smooth muscle cells and endothelial cells. This work illustrates the complex orchestration of lipid events that accompany the PIP_2 signal transduction pathway, and underscores the importance of future investigations to define the role of PLD and nonphosphoinositide-derived second messengers.

F 607 AUTOCRINE REGULATION IN THE GROWTH OF A HUMAN ESOPHAGEAL CARCINOMA CELL LINE. Shan-chun Chen[1,3], Chen-Kung Chou[1,2], Chungming Chang[1,2], 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. Epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1) and platelet-derived growth factor (PDGF) were potent growth promoting agents for a human esophageal carcinoma cell line CE48T/VGH. They stimulated 2.8- to 4-folds of cell proliferation in the serum-free condition, and induced transient expression of c-fos gene. In vitro tyrosine kinase assay revealed specific phosphotyrosine-containing proteins with molecular weights correlated with the reported EGF receptor, β-subuint of IGF-1 receptor, and PDGF receptor. Pretreatment of CE48T/VGH cells with TPA for 24 hr down-regulated protein kinase C (PK-C) and resulted in total abolishment of the proliferative response of PDGF. The response of EGF was inhibited to 70% by TPA pretreatment, while IGF-1 response was unaltered. Therefore, different pathways, PK-C dependent or PK-C independent, maybe involved in the stimulation of CE48T/VGH cell proliferation by EGF, IGF-1 and PDGF. Furthermore, expression of IGF-1 and PDGF genes were detected by the Northern analysis. Besides, basal cell growth was significantly inhibited by an addition of anti-PDGF Ab to the serum-free culture. Thus, an autocrine regulation may play a role in the growth of human esophageal carcinoma cells.

F 608 PROTEIN KINASE ACTIVITY OF THE INSULIN RECEPTOR IS ESSENTIAL FOR INSULIN REGULATED GENE EXPRESSION, C.K.Chou and S.C.Lin Department of Medical Research, Veternas General Hospital, Graduat Institute of Microbiology and Immunology, National Yang-Ming Medical College, Shih-Pai, Taipei, Taiwan, Republic of China.
Chinese hamster ovary cell line (CHO) and two stably transfected cell lines derived from CHO were used. CHO-wut2 and CHO-mut2 were transfeted with cDNA coding either wild type human insulin receptor or Lys 1018 substituted in ATP- binding domain of human insulin receptor a-subunit. The mutated insulin receptors expressed on CHO-mut2 bound insulin normally but no receptor tyrosine kinase activity in vitro and in vivo. Parental CHO, CHO-wt2 and CHo-mut2 cell lines express 2,000, 240,000 and 220,000 insulin receptors per cell respectivily. Plasmid DNA carrying chloramphenicol acetyl transferase (CAT) gene driven by P33 gene promoter, an insulin regulated gene promoter, was transfected into different CHO cell lines by calcium phosphate precipitation. Transfected cells were then incubated with different concentration of insulin for 24 hours and CAT activity were then assayed. CAT gene expression in CHO-wt2 cells showed 10-50 fold more sensitive to insulin than that in parental and CHO-mut2 cells. It is suggested that, the kinase negative receptors expressed on CHO-mut2 cells lost the ability to mediate insulin action on gene expression.

F 609 ANGIOTENSIN STIMULATES TYROSINE PHOSPHORYLATION IN RAT LIVER EPITHELIAL CELLS. H. Shelton Earp, William R. Huckle, Carol Prokop, and Bryan McCune, Lineberger Cancer Research Center, University of North Carolina, Chapel Hill, NC 27599. We have used a continuous line of non-transformed liver cells to demonstrate EGF-dependent and angiotensin-dependent accumulation of inositol phosphates. This indicates that both agents activate phospholipase C (PLC)in these cells. Simultaneous evaluation of phosphotyrosine (p-tyr) containing substrates was performed using affinity purified anti-p-tyr antibodies to immunoblot both RIPA lysates of intact cells and PLCγ immunoprecipitates. This procedure revealed that EGF stimulated tyrosine phosphorylation of several substrates including the EGF receptor and PLC-γ. Angiotensin did not stimulate tyrosine phosphorylation of either the EGF receptor or PLC γ. (Huckle, Hepler, Rhee, Harden and Earp, submitted). Surprisingly, angiotensin treatment of intact cells did stimulate the tyrosine phosphorylation of substrates at 125kDa, 75kDa and 66kDa. [Arg-8] vasopressin, another agent that increases intracellular calcium in these cells, had a similar effect on p-tyr content of p125, p75 and p66 as did the calcium ionophore, A23187. The hormones stimulated p-tyr accumulation within 15 seconds; the stimulation was diminished by 2 min. The effect of A23187 was delayed and prolonged. Preincubation of intact cells with vanadate (200 μm) enhanced the effect of angiotensin and A23187. The data suggest that a calcium-dependent process can activate tyrosine phosphorylation in an epithelial cell line.

F 610 TRANSFORMING GROWTH FACTOR-ß STIMULATES THE EXPRESSSION OF α_2-MACROGLOBULIN BY ADRENOCORTICAL CELLS,

Jean-Jacques Feige, De Li Shi, Catherine Savona, Jean Gagnon*, Claude Cochet, and Edmond M. Chambaz, Laboratoire de Biochimie des Régulations Cellulaires Endocrines and *Laboratoire de Biologie Structurale, DRF/LBIO, Centre d'Etudes Nucléaires, Grenoble, FRANCE

Adrenocortical cell major secreted protein was purified from the conditioned medium of primary cultures of bovine adrenocortical (BAC) cells. Immunochemical analysis and N-terminal sequencing of the purified protein identified it to α2-macroglobulin (α2-M). Study of α2-M production by BAC cells revealed that its secretion was stimulated several fold by TGFß1 in a time-dependent (reaching a plateau at 24 h) and dose-dependent (ED_{50}=0.1ng/ml $TGF\beta_1$) manner. It was blocked when BAC cells were exposed to DRB, a potent inhibitor of RNA polymerase II, suggesting that TGFß1 was acting at the transcriptional level. Northern blot analysis confirmed that the α2-M mRNA level was increased 4 fold in BAC cells following TGFß1 treatment. Given the previous reports that TGFß1 is a potent inhibitor of adrenocortical steroidogenesis (Feige J.J. et al., 1987, J. Biol. Chem. **262**, 13491-13495) and that α2-M is a TGFß1-binding protein, these observations suggest that α2-M may play an important role in the homeostatic regulation of adrenocortical functions.

F 611 THE HUMAN INSULIN-LIKE GROWTH FACTOR-II (IGF-II) / MANNOSE-6-PHOSPHATE (M6P) RECEPTOR IS VARIABL Y EXPRESSED IN MULTIPLE TISSUES, B. Funk, U. Kessler, W. Eisenmenger, W. Kiess, University of Munich, FRG
The rat IGF-II/M6P receptor is expressed in multiple tissues. In the rat, the receptor is developmentally regulated (Proc.Natl.Acad.Sci. 84:7720 (1987); J.Biol.Chem., in press). In this study we measured the IGF-II/M6P receptor in human tissues from 23 weeks gestation through two years postnatal. Tissues from aborted or stillborn fetuses and infants who died suddenly were homogenised in buffer containing 2% Triton-X-100, boiled in Laemmli buffer containing 2% SDS and the extract stored at -20°C after being kept overnight at 4°C. Aliquots of the protein extracts (0.2 mg) were analysed by SDS-PAGE and the protein bands were transfered onto nitrocellulose. Immunoblotting was performed using anti-IGF-II/M6P receptor serum (#66416). Ligand blotting was performed using 125-I-IGF-II and subsequent autoradiography. IGF-II/M6P receptor was expressed in all tissues examined. Receptor content was highest in thymus, heart, spleen and kidney, intermediate in liver, lung and testis and lowest in brain and muscle. The apparent M_r of the receptor is 215,000 and varied among the organs studied. Receptor content in the different organs did not vary in a developmental fashion. We conclude that the human IGF-II/M6P receptor is not developmentally regulated from 23 weeks gestation through two years postnatal but variabl y expressed in all organs examined. (Supported by DFG, Ki-365/1-1)

F 612 PROTEIN KINASE C ISOFORMS IN MDCK CELLS : REGULATION BY PHORBOL ESTER AND HORMONES. Catherine Godson, Barbara A. Weiss and Paul A. Insel. Dept. Pharmacology M-036, UCSD, La Jolla, CA 92093.The recently described heterogeneity of the protein kinase C (PKC) gene family suggests that different isoforms of the enzyme may be activated differentially by hormones within a given tissue or subcellular compartment and that phospholipid metabolites such as diacylglycerol and arachidonic acid which may be produced sequentially in response to cell surface stimulation may differentially activate PKC isoforms. To investigate this phenomenon we have identified the PKC isoforms present in Madin Darby Canine kidney (MDCK) cells and their potential role in hormone (bradykinin [BK] and epinephrine [epi]) stimulated arachidonate release. Using sequence specific antipeptide antibodies (Makowske, M. et al., [1988] J. Biol. Chem. 263 : 3402-3410) in Western blotting procedures we have identified Type II (ß) and Type III (α) PKC in MDCK cells. We have found that down regulation of PKC by phorbol dibutyrate treatment completely inhibits epi and phorbol ester stimulated arachidonate release whereas BK stimulated arachidonate release is reduced but not absent. PKC activity, as determined by an exogenous histone phosphorylation assay is decreased but not absent in these cells. Western blotting indicates (i) preferential down regulation of type III PKC under these conditions and (ii) preferential translocation of type III PKC by phorbol ester under conditions in which arachidonate activity is stimulated. Work is currently underway to define the types of PKC translocated by epi and BK stimulation and their specific cofactor requirements. The data obtained thus far suggest that different PKC isoforms may be involved in different regulation of Phospholipase A2 by hormone and phorbol esters.

F 613 POSSIBLE IDENTITY OF THE RECEPTORS AND BIOLOGICAL ACTIVITIES OF MELANOMA GROWTH STIMULATORY ACTIVITY (MGSA/GRO) AND INTERLEUKIN 8, J.H. Han[1], H.G. Thomas[1], A.K. Samanta[2], C.O.C. Zachariae[2], E. Balentien [1,3], R.Derynck[3], K. Matsushima[2], A. Richmond[1] [1]Veterans Administration Medical Center/Vanderbilt University, Nashville, TN 37232 [2]Laboratory of Molecular Immunoregulation, NCI, Frederick, MD 21701-1013 and [3]Department of Molecular Biology, Genentech, Inc., S. San Francisco, CA 94080.
Melanoma growth stimulatory activity (MGSA/gro) is a growth related protein from the B-thromboglobulin (B-TG) superfamily. Another member of the B-TG family, interleukin-8 (IL-8), is chemotactic for neutrophils/lymphocytes and stimulates hydrogen peroxide production in neutrophils. Both genes map to the same chromosomal region and are transcriptionally activated by many of the same factors. MGSA competes with ^{125}I-IL-8 for binding to neutrophil IL-8 receptors. MGSA exhibits neutrophil chemotactic activity equivalent to that of IL-8 and Il-8 stimulates the ^{3}H-thymidine incorporation in low density serum free cultures of Hs294T melanoma cells. When the MGSA receptors/binding proteins were characterized by MGSA ligand affinity chromatography, the receptor/binding proteins were in the same molecular weight range as the IL-8 receptor(s), 65,000 and >200,000 Mr. However, the effective doses for MGSA bioactivity varied. These factors are capable of acting on a broad range of target cells and therefore are potential mediators of the wound healing/inflammatory response.

F 614 OKADAIC ACID DIFFERENTIALLLY REGULATES INSULIN ACTION IN CULTURED LIVER, FAT, AND MUSCLE CELLS, Susan Hess, Craig Suchin, and Alan Saltiel, Department of Molecular Oncology, Rockefeller University, New York, New York 10021.
The molecular mechanisms of insulin action have remained elusive. One of the barriers in elucidating insulin action is its pleiotropic nature. We have utilized the specific protein phosphatase inhibitor, okadaic acid, to differentiate between different actions of the hormone. In cultured H-35 hepatoma cells and 3T3-L1 adipocytes, okadaic acid inhibits the stimulation of lipid synthesis by insulin. Similarly, in H-35 hepatoma cells, okadaic acid inhibits the stimulation of glycogen synthesis by insulin. Interestingly, the apparent half-maximal effective concentration for these two actions differ; Insulin-stimulated lipogenesis is inhibited with an EC_{50} of 50 nM okadaic acid, whereas insulin stimulated glycogen synthesis is inhibited with an EC_{50} of approximately 400 nM okadaic acid. These data suggest that different protein phosphatases may be responsible for the actions of insulin. In contrast to the acute anabolic effects of the hormone, the stimulation of glucose and amino acid uptake was unaffected by okadaic acid in H-35 hepatoma cells or BC3H1 myocytes. These findings indicate that the cellular actions of insulin are mediated through different pathways, and that the acute promotion of lipid and glycogen synthesis involves different protein phosphatases.

F 615 REGULATION OF LUNG EPITHELIAL CELL PROLIFERATION BY TRANSFORMING GROWTH FACTORβ1. P.H. Howe and E.B. Leof, Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37232.
Transforming growth factorβ1 (TGFβ1) inhibits DNA synthesis and cell division in mink lung epithelial cells (CCL64) through as yet undetermined mechanisms. While the effect of TGFβ1 on expression of genes believed to initiate cell cycle traverse has received the most attention, in this study we present data which indicate that TGFβ1 inhibition of early G0/G1 events is not sufficient for growth inhibition. Addition of 10% fetal bovine serum (FBS) and 20 ng/ml epidermal growth factor (EGF) to density-arrested CCL64 cells initiates cell cycle traverse and results in maximum DNA synthesis. These cells enter S phase following a minimum G1 of approximately 12 hours with maximal DNA synthesis occurring 24 hours after FBS + EGF stimulation. Addition of TGFβ1 to stimulated cells results in a dose-dependent decrease in the incorporation of ^{3}H-thymidine into DNA. TGFβ1 when added at any time during the 12 hour G1 period inhibits DNA synthesis to a similar extent (approximately 80-100%) as when it is added at the time of stimulation. If cultures progress beyond commitment to DNA synthesis (i.e. > 12 hours following stimulation with FBS +EGF) before TGFβ1 is added, then no growth inhibition is observed. Kinetic analyses, based both on ^{3}H-thymidine incorporation and autoradiography, demonstrate that TGFβ1 addition to stimulated cultures blocks the cells at the G1/S phase boundary, and that removal of TGFβ1 from the culture media results in the cells entering S phase within 1-2 hours. Entry into DNA synthesis following TGFβ1 arrest is dependent on protein synthesis but independent of RNA synthesis. In cycling or rapidly growing cell cultures, TGFβ1 addition results in the arrest of the cultures in two distinct phases of the cell cycle. One population of cells arrests at the G1/S phase as described above, while the second population arrests prior to cell division in G2/M.

F 616 CLONING OF A cDNA ENCODING THE MOUSE IL-3 RECEPTOR, Naoto Itoh[1], Shin Yonehara[2], Jolanda Schreurs[1], Dan Gorman[1], Kazuo Maruyama[1], Huey-Mei Wang[1], Ai Ishii[2], Ichiro Yahara[2], Ken-ichi Arai[1], and Atsushi Miyajima[1], [1]DNAX Research Institute and [2]Tokyo Metropolitan Institute. Mouse IL-3 binds to the IL-3 receptor with high and low affinity, induces tyrosine phosphorylation and stimulates proliferation and differentiation of hemopoietic cells. Using the putative antiIL-3 receptor antibody, anti-Aic2, we have isolated a cDNA encoding the IL-3 receptor from an expression library made from an IL-3 dependent mouse mast cell line. The full length cDNA encodes for 878 amino acids including the signal sequence. A single hydrophobic membrane spanning domain separates the extracellular and the intracellular domains. Fibroblasts transfected with the cloned cDNA binds IL-3 with low affinity. No consensus sequence for a tyrosine kinase is present in the cytoplasmic domain. These results indicate that the functional high affinty receptor requires additional molecule(s). A sequence comparison of the IL-3 with other cytokine receptors reveales a common motif, suggesting the existence of a new receptor gene family.

F 617 A C-TERMINAL ELEMENT SUPPRESSES INTRINSIC ACTIVITIES OF THE INSULIN RECEPTOR, Jeffrey D. Johnson, Martine Aggerbeck and William J. Rutter, Hormone Research Institute. University of California, San Francisco, CA 94143

To determine the role of the C-terminal tail of the human insulin receptor (hIR) in the mediation of the biological effects of insulin we have produced and characterized cell lines expressing mutant receptors truncated or substituted within this region. The largest truncation (in IRd95) removes 95 out of the 98 amino acids C-terminal to the tyrosine kinase domain. A second deletion mutant (IRd30) lacks the 30 C-terminal amino acids including two principal tyrosine autophosphorylation sites. In a third mutant hIR we have substituted the C-terminal tail of the hIGF1 receptor in place of that of the hIR (IR-IGRt). Each of the mutant receptors binds insulin with affinity identical to the wild type receptor. Cells bearing the IRd30 or IR-IGRt receptors, but not the IRd95 receptor, retain the capacity for autophosphorylation in vitro and in vivo as well as endogenous substrate phosphorylation. The IRd30 and IR-IGRt receptors can mediate insulin dependent increases in glucose uptake and c-fos induction. The IRd30 and IR-IGRt, but not the IRd95, receptors produce elevated basal rates of glucose uptake and also result in the expression of unusual transcripts of c-fos. These results indicate that at least a portion of the insulin receptor C-terminal tail is required for biological function and that more modest deletion or substitution can restore some of the properties of the wild type receptor but result in alteration of the biological properties of the receptor in the absence of insulin. These results imply the presence of an element within the IR C-terminus which participates in suppressing specific biological functions intrinsic to the hIR.

F 618 PHOSPHATIDYLINOSITOL-3 KINASE IS ACTIVATED BY INSULIN, Rosana Kapeller Neil B. Ruderman, Morris F. White and Lewis C. Cantley, Department of Physiology, Tufts University School of Medicine, Boston, MA 02111. The function of cellular targets of the insulin receptor are unknown. A phosphatidylinositol-3 kinase (PI-3 kinase) has been recently shown to associate with several protein-tyrosine kinases and its activation can be correlated with cell growth. This enzyme phosphorylates the D-3 position of the inositol ring to produce polyphosphoinositides that are not in the pathway that generates inositol-1,4,5-triphosphate. Here we report that PI-3 kinase activity is immunoprecipitated from insulin stimulated CHO cells by anti-phosphotyrosine and anti-insulin receptor antibodies; insulin concentrations as low as 0.3nM produced this effect within one minute.CHO/IR cells expressing the wild type human insulin receptor (80,000 receptors/cell) showed a much greater insulin dependent PI-3 kinase activity than did control CHO cells (2,0000 receptors/cell).In vivo, the PI-3 kinase lipid products that were marginally detected in unstimulated cells,increased markedly after one minute stimulation by insulin. Furthermore, insulin elicited the appearance of several [^{35}S]-methionine labeled proteins in the anti-insulin receptor immunoprecipitates which coincide with PI-3 kinase activity in the same immunoprecipitates. These results suggest that insulin regulates a PI-3 kinase through a physical association between the insulin receptor and the PI-3 kinase and/or by tyrosyl phosphorylation.

F 619 GENETIC AND BIOCHEMICAL EVIDENCE THAT AUTOPHOSPHORYLATION OF THE PDGF RECEPTOR IN THE KINASE INSERT REGION REGULATES INTERACTIONS WITH CELLULAR PROTEINS. Andrius Kazlauskas and Jonathan A. Cooper, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

We have shown that the the major site of phosphorylation of the β subunit of the human PDGF-R is Tyr-857. Tyr-751, which lies within the kinase insert region, is a less prominent site in vivo but the major site in vitro. We mutated each of these tyrosine residues and expressed the corresponding cDNAs in epithelial cells lacking endogenous PDGF-Rs. The incorporation of ^{3}H-thymidine following exposure to PDGF was dramatically increased in cells expressing the wild-type (WT) PDGF-Rs, but not in cells expressing an empty vector. The mitogenic responses of cells expressing the Tyr-857 and Tyr-751 mutant were reduced to 48% and 4% of WT, respectively. Preliminary data suggest that tyrosine phosphorylation of cell proteins may be reduced by mutation of Tyr-857, but is unaffected by mutation of Tyr-751. Immunoprecipitates of wild-type PDGF-Rs prepared from PDGF-treated cells contained a phosphatidylinositol (PI) kinase activity as well as three polypeptides (120, 84 and 72 KDa) that could be phosphorylated in vitro, presumably by the PDGF-R. Studies with mutant receptors showed that these associations required both kinase activity and a tyrosine residue at position 751. Mutation of Tyr-857 had no effect. Consistent to the situation in a living cell, the in vitro binding of the 120 KDa protein and the PI kinase was dependent on phosphorylation of Tyr-751. These data suggest that autophosphorylation of Tyr-751 in the kinase insert region triggers the association of the activated PDGF-R with specific cellular proteins, including a PI kinase, and that these events are critical for the relay of a mitogenic signal.

F 620 EFFECT OF TGF-ß ON TYROSINE PHOSPHORYLATION IN IL-3-DEPENDENT AND ONCOGENE-INFECTED FACTOR-INDEPENDENT MYELOID PROGENITOR CELL LINES. [1]J.R. Keller, [2]K.T. Sill, [3]G. K. Sing, [4]D.K. Ferris and [5]F.W. Ruscetti. [1,2,4]BCDP-PRI, [3,5]LMI-BRMP, NCI-FCRF, Frederick, MD. We have previously shown that transforming growth factor-ß (TGF-ß) is a selective inhibitor of colony stimulating factor-driven hematopoietic cell growth. In addition, TGF-ß inhibits the growth of interleukin-3 (IL-3) -dependent and independent murine myeloid leukemic progenitor cell lines. IL-3 stimulates the phosphorylation of various proteins in these factor dependent cell lines. Since protein phosphorylation occurs during transduction of a proliferative signal, we studied whether TGF-ß inhibition affects tyrosine phosphorylation. Using monoclonal antibodies to phosphotyrosyl proteins these proteins were analyzed by both single and two-dimensional electrophoresis. Our results indicate that TGF-ß inhibits tyrosine phosphorylation of multiple proteins both in IL-3-dependent and independent cells established by infection with virus containing oncogenes. These effects on tyrosine phosphorylation were both time and concentration dependent at physiological levels of TGF-ß suggesting that TGF-ß effects signal transduction through protein phosphorylation.

F 621 **ARP-1, A NOVEL MEMBER OF THE NUCLEAR RECEPTOR SUPERFAMILY THAT BINDS TO THE REGULATORY SEQUENCES OF THE APOLIPOPROTEIN AI GENE.** **John A. A. Ladias and Sotirios K. Karathanasis, Department of Cardiology, Harvard Medical School, Boston, MA 02115.**

In mammals the gene coding for apolipoprotein AI (ApoAI), a plasma protein involved in lipid metabolism, is expressed primarily in liver and intestine. We have previously shown that the -238 to -41 DNA region 5' to the transcriptional start site of the ApoAI gene functions as a hepatoma-cell (HepG2) specific transcriptional enhancer. Nuclear extracts from various tissues and cell types contain a protein, designated ApoAI Regulatory Protein-1 (ARP-1), that binds specifically to -223 to -192 DNA region of the ApoAI gene and is involved in the regulation of its expression. Here we report the isolation and characterization of a cDNA clone encoding ARP-1 from a human λgt11 expression library. The cDNA-derived amino-acid sequence of ARP-1 contains a two-Zn-finger motif remarkably similar to that of members of the steroid/thyroid hormone receptor superfamily. The amino-terminal domain of ARP-1 contains proline-rich and glutamine-rich streches characteristic of transcription activating domains of other regulatory proteins. To date, we have not identified a ligand for ARP-1. Preliminary co-transfection experiments of the ARP-1 cDNA clone and the CAT reporter gene driven by the ApoAI hepatoma-specific enhancer into HepG2 cells, show that ARP-1 supresses the expression of the reporter gene in a dose-dependent manner. In vitro-translated ARP-1 can bind specifically to regulatory elements of other genes such as the chicken ovalbumin, rat insulin II, human apolipoprotein C-III and apolipoprotein B, suggesting that it may be involved in the regulation of their expression. Finally, ARP-1 can also bind to the thyroid-hormone responsive element, raising the possibility for its involvement in thyroid-hormone responsiveness.

F 622 OVEREXPRESSION OF PROTEIN KINASE C-α SUBTYPE IN SWISS/3T3 CELLS REDUCES BOTH HIGH AND LOW AFFINITY RECEPTOR NUMBERS FOR EGF, Etta Livneh, Hagit Eldar, +Axel Ullrich and Yaffa Zisman. Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel. +Genentech Inc. South San Francisco, CA, USA.
The full length cDNA coding for the α form of PKC_α (PKC) was introduced into Swiss/3T3 cells using a retroviral expression system. This has enabled the generation of a series of cell lines stably expressing high levels of PKC_α enzyme. PKC_α-over-producing cell lines exhibited enhanced growth rates and reached higher saturation densities than parental Swiss/3T3 cells. However, stimulation of DNA replication in these cell lines by either serum, EGF, PDGF or phorbol esters (TPA), was markedly reduced or even abolished. Scatchard plot analysis of EGF binding to EGF receptors expressed on the cell surface of PKC_α-producing cells has revealed reduction in receptor numbers without change in the affinities for EGF of the remaining receptors. Therefore, we suggest that reduced receptor numbers may be responsible for this diminished mitogenic response. Furthermore, we provide evidence that reduced EGF-receptor numbers in PKC_α-overproducing cells may result both from the reduced biosynthesis rates of EGF receptor molecules and the enhanced loss of their receptors from the cell surface. Our studies show that deregulation of PKC_α, i.e. by increasing its expression level in the cell, affects in turn cell metabolism and the cell surface EGF-receptor numbers. These phenotipic changes are maintained in a permanent fashion, even in the absence of an exogenous activator of PKC_α. Similar molecular mechanism that may underlay neoplasia and tumor promotion will be discussed.

F 623 A 372 BP FRAGMENT 5' OF THE IL2 GENE CONFERS INHIBITION BY GLUCOCORTICOIDS OF A LINKED REPORTER GENE Petri S. Mattila ', Steve Fiering ', Gerald Crabtree* and Leonard A. Herzenberg '. Department of Genetics ' and Howard Hughes Medical Institute, Department of Pathology *, Stanford University School of Medicine, 94305 Stanford, CA. The mechanism by which glucocorticoids inhibit interleukin 2 (IL2) expression is unknown. To study this mechanism a glucocorticoid sensitive mouse T cell line, LBRM33.1A5, was stably transfected with enhancer regions from the human IL 2 gene controlling transcription of a *E.coli* lacZ reporter gene. A fragment of the IL2 gene from -326 to +45 (enhancer and promoter) responded to the physiological signals known to induce IL2. The cells were stimulated with 2uM ionomycin and 50 ng/ml phorbol myristate acetate (PMA) and the B-gal activity was measured 8 hours after stimulation using a fluorogenic, individual viable cell assay (Nolan et al. PNAS 85, 2603-2607, 1988) and a conventional enzyme assay which measures B-gal activity in extracts of viable sorted cells. 100 nM dexamethasone caused a 20-fold inhibition of B-gal activity in stimulated cells that were stably transfected with the 372 bp enhancer and promoter region controlling lacZ expression. No significant inhibition of lacZ expression was observed in stably transfected cells that had one of the IL 2 enhancer sites, nuclear factor of activated T cells (NF-AT), in 3 tandem copies attached at -70 bp (the bare Il 2 promoter) with lacZ attached at +45. The results indicate that glucocorticoids inhibit the transcription of IL 2 through the 372 bp region and that this is not mediated by the NF-AT binding site. Further, expression from the NF-AT construct is inhibited by cyclosporin A, therefore it uses a mechanism distinct from the inhibition by glucocorticoids. The 20-fold inhibition observed is markedly more pronounced than the 3 to 4 fold inhibition previously reported with other unrelated enhancers that are negatively regulated by glucocorticoids. We are now looking at glucocorticoid activity with other enhancer sites replacing NF-AT.

F 624 Characterization of NGF-stimulated Microtubule-associated Protein kinase from PC12 Cells, T. Miyasaka, P. Sherline, M. V. Chao, and A. R. Saltiel, Laboratory of Molecular Oncology, Rockefeller University, and Department of Cell Biology, Division of Hematology/Oncology, Cornell University Medical School, New York, New York 10021. Although the precise molecular events in NGF action remain uncertain, numerous studies indicate an important role for protein phosphorylation. We have identified an NGF-stimulated MAP kinase from PC12 cells. The kinetic and physicochemical properties of this protein kinase are identical to the insulin-stimulated MAP kinase identified in 3T3-L1 adipocytes. NGF causes a rapid activation of this enzyme (2-5 min) followed by a slower activation at 30-60 min. Pretreatment of activated MAP kinase with alkaline phosphatase reduces activity, suggesting a mechanism of activation requiring phosphorylation. A phosphoprotein with an apparent molecular mass of 42 kDa was copurified with MAP kinase activity. Both activation of MAP kinase and phosphorylation of the 42 kDa protein are stimulated by NGF and inhibited by staurosporine. These findings suggest that the early phase activation of MAP kinase by NGF may be attributed to a receptor-associated, staurosporine-sensitive tyrosine kinase.

F 625 DOWN-REGULATION OF THE PDGF RECEPTOR BY THE NON-PHORBOL ESTER TUMOR PROMOTER OKADAIC ACID. Lawrence J. Mordan, Richard Honkanen, Alton L. Boynton, and Nicholas M. Dean, Basic Science Program, Cancer Research Center of Hawaii, University of Hawaii at Manoa, Honolulu, HI 96813.

Okadaic acid (OA), a polyether compound isolated from the marine sponge Halichondria okadai, is a potent inhibitor of types 1 and 2A phosphatases and a very effective non-phorbol ester tumor promoter in the mouse skin model. We have found that OA inhibits PDGF-stimulated DNA synthesis in density-arrested C3H/10T1/2 mouse fibroblasts and, consequently, have examined the effects of OA on PDGF receptor function. OA decreases PDGF binding sites in a dose- (IC_{50}=26nM) and time- dependent manner. OA inhibits the PDGF-induced rise in free intracellular Ca^{2+} and phosphatidyl inositol hydrolysis. Because OA inhibits phosphatase activity in these cells (IC_{50}=10nM), these results strongly suggest that phosphatases play a critical role in regulating PDGF receptor function and, subsequently, the responsiveness of cells to this mitogenic growth factor.

F 626 **SOLUBILIZATION AND CHARACTERIZATION OF THE RECEPTOR FOR THE NEUROPEPTIDE GASTRIN-RELEASING PEPTIDE (BOMBESIN),** Luigi Naldini[1,3], Daniela Cirillo[1,3], Terry W. Moody[2], Paolo M. Comoglio[3], Joseph Schlessinger[1] and Richard Kris[1]. [1]Rorer Biotechnology Inc., 680 Allendale Road, King of Prussia, PA 19406, U.S.A. [2]Department of Biochemistry, the George Washington University, Washington, D.C., U.S.A. [3]Department of Biomedical Sciences & Oncology, C.so Massimo D'Azeglio 52, 10126 Torino, Italy

The receptor for the neuropeptide gastrin releasing peptide (GRP), the mammalian homologue of bombesin, was solubilized from rat brain and Swiss 3T3 cells using the zwitterionic detergent 3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonic acid (CHAPS) and the cholesteryl hemisuccinate ester (CHS). Only the combination of the detergent CHAPS and the cholesteryl ester CHS in a glycerol containing buffer satisfactorily preserved the binding activity upon solubilization. Physico-chemical, kinetic and equilibrium-binding parameters were evaluated both for the membrane-bound and the soluble receptor. The solubilized receptor was then characterized biochemically. Swiss 3T3 membranes were covalently labelled with [^{125}I]GRP and homobifunctional crosslinkers, solubilized and analyzed under non denaturing conditions by molecular sieving chromatography and sucrose density gradient sedimentation. The protein bound radioactivity was resolved in two different peaks, a major one of apparent molecular weight 220,000 (peak 1) and a minor one of 80,000 (peak 2). SDS-PAGE analysis showed that the previously described 75 kD protein was present at the major [^{125}I]GRP labelled species in both peaks. Pretreatment of the fractions containing the larger size peak 1 with boiling SDS prior to rechromatography caused a shift of the protein bound radioactivity to the 80,000 molecular weight peak 2 plus the appearance of an additional smaller peak of apparent molecular weight 30,000. A soluble receptor assay was performed on fractions collected from molecular sieving chromatography of solubilized membranes. Specific ligand binding activity eluted with a volume corresponding to peak 1. These results indicate that the active form of bombesin/GRP receptor is a large complex containing the 75 kD binding domain. The involvement of a GTP responsive protein and/or a protein tyrosine kinase was also investigated.

F 627 STUDIES ON THE STRUCTURE AND REGULATION OF THE HEPATIC INTERLEUKIN-6-RECEPTOR

Stefan Rose-John, Heidi Schooltink, Erich Hipp, Dorothée Lenz, Gabi Dufhues,

One of the major molecular events that initiate the hepatic acute phase response to injury and infection is the binding of hepatocyte stimulating factor / interleukin-6 (IL-6) to its surface receptor on hepatocytes. The binding of iodinated rhIL-6 to this receptor has been studied with isolated plasma membranes from rat liver. A single binding affinity with a K_D of 1.3×10^{-12} M was deduced and a number of 1500 receptors per liver cell has been estimated. Affinity cross-linking of iodinated rhIL-6 to either rat liver plasma membranes or to human hepatoma cells (HepG2) allowed the detection of an IL-6 binding polypeptide with a molecular mass of 80 kDa.

Treatment of HepG2 cells with dexamethasone led to a time- and dose-dependent upregulation of IL-6-receptor mRNA. Under conditions of IL-6-receptor upregulation by dexamethasone the induction of the acute phase protein fibrinogen at the mRNA level is much stronger and occurs earlier than without dexamethasone. We therefore propose that the expression levels of IL-6-receptor might be a rate limiting step in acute phase protein induction.

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F 628 **DELETION ANALYSIS OF THE INSULIN RECEPTOR EXTRACELLULAR DOMAIN,** Erik Schaefer, Kenneth Siddle*, and Leland Ellis, Howard Hughes Medical Institute and the Department of Biochemistry, UT Southwestern, Dallas, TX and the *Department of Clinical Biochemistry, University of Cambridge, UK. We have previously demonstrated that both extracellular and cytoplasmic domains of the insulin receptor (IR) can be independently expressed as soluble proteins that are capable of autonomous function (high affinity insulin binding and protein-tyrosine kinase activity, respectively). To explore the structural and functional organization of the soluble extracellular domain protein (designated hIR01) we have engineered and expressed in a heterologous cell system a series of truncated hIR01 proteins and assessed the efficiency of secretion, stability and interaction of each protein with both the polypeptide hormone insulin and a panel of monoclonal antibodies that are directed against eight distinct epitopes of the IR ectodomain. The results of this analysis have now defined specific sites within the primary sequence of the IR ectodomain that demarcate boundaries at which truncation generates subdomains of hIR01 that are capable of folding de novo during biosynthesis into stable proteins that are synthesized and secreted with wild-type kinetics, and which retain the epitopes required for the interaction with a subset of two conformation specific monoclonal antibodies. The oligomeric nature of these truncated proteins and their interaction with insulin is under investigation. These results provide us with important structural landmarks within the primary sequence of the IR extracellular domain (which are not obvious from perusing the primary sequence, super-secondary structure predictions, or the exon-intron boundaries alone) that will guide us in further analysis of the biochemistry of this complex domain of the IR.

F 629 HORMONE-STIMULATED PHOSPHOLIPASE A_2 IN PLANTS GENERATES LPC AS BIOLOGICALLY ACTIVE LIPID TO STIMULATE MEMBRANE-ASSOCIATED PROTEIN KINASE. Günther F.E. Scherer, Bernadette André and Georg Martiny-Baron, Botanisches Institut, Universität Bonn, D-5300 Bonn 1, FRG. In cultured soybean cells (Glycine max L.) PC and PE were predominantly labeled by ^{14}C-choline and ^{14}C-ethanolamine, respectively. After application of $5x10^{-4}$ M of the plant growth hormone auxin a shift of label from PC to LPC and from PE to LPE was observed within 1 min. When the hormone was removed by washing the in LPC accumulated label decreased again. Only the native auxin IAA and active analogues were effective in stimulating this reaction whereas inactive analogues were not. The physiologically antagonizing plant hormone abscisic acid decreased the effects of auxin. In summary, a hormone-stimulated phospholipase A_2 in plant cells is indicated. With isolated prelabeled membranes the stimulation of endogenous PLA_2 was observed at $2x10^{-5}$ M hormone concentration by measuring the accumulation of of LPC or LPE in the membranes. The pH optimum of this reaction is very similar to the pH optimum for hormone binding of the plasma membrane binding site. In the presence of GDP auxin does not stimulate PLA_2 whereas in the presence of GTPγS auxin is active, indicating a function of G-proteins in this pathway. LPC, LPE and the chemically similar platelet-activating factor but neither phorbol ester nor DAG and/or PS activate membrane-associated protein kinase in plants. This lysophospholipid-activated protein kinase phosphorylates a number of polypeptides in tonoplast and plasma membranes one of which has been identified as the regulatory subunit of vacuolar H^+-ATPase. We postulate a novel signal transduction chain in plants consisting of signal(auxin) - receptor(?) - G-protein - phospholipase A_2 - LPC/LPE - protein kinase - regulated enzymes(H^+-ATPases) where LPC/LPE are second messengers.

F 630 **gp185erbB-2 and EGFR-activated mitogenic pathways: differences and similarities.** O. Segatto*, F. Fazioli*, J.H. Pierce*, F. Lonardo*, D. Wexler*, M. Ruggiero*, Sue Goo Rhee+, S.A. Aaronson* and P.P. Di Fiore*. *LCMB, NCI, Bethesda, MD and +NHLBI, Bethesda, MD
The epidermal growth factor (EGF) receptor and *erb*B-2 genes encode structurally related proteins whose overexpression has been implicated in the pathogenesis of certain human malignancies. The extensive homology between *erb*B-2 and EGFR prompted us to compare their respective biological actions and underlying signalling pathways. We, therefore, analyzed the interaction of the *erb*B-2 tyrosine kinase with phospholipase C-γ (PLC-γ) and its effects on the PIP_2 breakdown pathway. Our results show that PLC-γ is a substrate for the *erb*B-2 tyrosine kinase, similar to what has been demonstrated for the EGFR. No quantitative difference was detected in the ability of EGFR and *erb*B-2 to co-immunoprecipitate PLC-γ and to induce its tyrosine phosphorylation. In addition, the magnitude of the increase in inositol phosphates formation induced by activation of the EGFR and *erb*B-2 kinases was comparable. Since *erb*B-2 is 100 fold more active as a transforming gene than EGFR, we conclude that while phosphorylation of PLC-γ may be an important event in the activation of the *erb*B-2 specific mitogenic pathway, it does not account for *erb*B-2 transforming potency as compared to EGFR.

F 631 STRUCTURE AND FUNCTION OF THE INTERLEUKIN-1 RECEPTOR, John Sims, Byron Gallis, Benson Curtis, William Fanslow, Karol Bomsztyk, and Steve K. Dower, Immunex Corp., Seattle, WA 98101.

The IL-1 receptor expressed on murine T cells and fibroblasts is a single polypeptide chain of 80,000 MW (about 20% of which is carbohydrate) which binds both IL-1α and IL-1β with a Ka of $\approx 3 \times 10^9$ M^{-1}. We have isolated cDNA clones of this receptor from the T cell lymphoma line EL-4 using a direct expression technique. The sequence of these clones shows that the receptor consists of an N-terminal ligand binding portion, a single transmembrane segment and a relatively large cytoplasmic domain. The recombinant receptor, when expressed in CHO cells, is fully functional in signal transduction.

The extracellular portion of the receptor is comprised of three immunoglobulin-like domains, all of which are required for ligand binding. A truncated receptor molecule expressing only the extracellular portion is soluble in aqueous solution and binds both IL-1α and IL-1β with the same affinity as does the natural, cell-bound receptor. The soluble form of the receptor is capable of preventing cell proliferation and lymph node enlargement in mice in response to injection of allogeneic cells, and greatly prolongs the life of cardiac allografts.

The cytoplasmic portion of the receptor is required for signal transduction. Although from its sequence the receptor is clearly not a kinase, one of the early intracellular events which follows IL-1 binding is phosphorylation of several proteins, one of which is the receptor itself. Studies to be discussed suggest that phosphorylation of the IL-1 receptor may account, at least in part, for the large difference between the level of IL-1 required for maximal biological responsiveness vs. that required for maximal receptor occupancy.

The receptor we have cloned is the principal if not the only receptor for IL-1 expressed on T cells and fibroblasts. Other cells, in particular B cells, appear to express a different IL-1 receptor polypeptide. The current state of evidence for this will be presented.

F 632 THE ERYTHROPOIETIN RECEPTOR REQUIRES PROTEIN KINASE C FOR TRANSDUCTION OF SIGNALS TO THE NUCLEUS. Rudolph Spangler, Steven Bailey, Arthur Sytkowski. New England Deaconess Hospital, Harvard Medical School, Boston, MA 02215.

The binding of erythropoietin (Epo) to its cell-surface receptor causes a rapid increase in c-myc mRNA levels in responsive cell lines and normal spleen cells. While necessary for c-myc activation, C kinase is not sufficient, because no single C kinase agonist induced c-myc. The Epo-induced increase in c-myc is sensitive to a pair of kinase inhibitors (H7 and H8) with a profile indicative of a signal transduction pathway which requires kinase C - i.e, H7 is 3X more potent than H8 at blocking Epo-induced c-myc mRNA accumulation. The C kinase inhibitors sphingosine, sangivamycin, and staurosporine blocked the Epo-induced signal at concentrations consistent with inhibition of C kinase. Finally, both Epo and the C kinase agonist TPA caused increased phosphorylation of the same major 80 kDa C kinase substrate, as visualized on 2-D gels. Taken together, these data indicate that 1) binding of Epo leads to activation of protein kinase C, 2) that this activation event is necessary in order for the signal to reach the nucleus, and 3) that at least one other event initiated by the receptor is required for transduction of the signal to the nucleus.

F 633 IN VITRO TYROSYL PHOSPHORYLATION OF GROWTH HORMONE (GH) RECEPTORS, S.E. Stred, J.R. Stubbart, C. Carter-Su, Dept. of Physiology, Univ. of Michigan, Ann Arbor, MI 48109. Our laboratory has shown that binding of GH to intact 3T3-F442A fibroblasts promotes phosphorylation of the GH receptor on tyrosyl residues. To investigate whether the receptor itself possesses kinase activity, an in vitro phosphorylation assay has been developed using GH-receptor complexes partially purified from these cells using anti-GH antiserum. When immunoprecipitated receptors from GH-treated cells are incubated with [γ^{32}P]-ATP in the presence of Mn^{2+} for 10 min at 30°C, ^{32}P is incorporated into a 121kDa phosphoprotein, solely on tyrosyl residues. The 121K phosphoprotein can be reprecipitated with a specific polyclonal anti-GH receptor antibody (kindly provided by W. Smith and F. Talamantes, U.Calif., Santa Cruz), indicating that pp121 is the GH receptor. In the presence of 5.5mM Mn^{2+}, maximal ^{32}P incorporation occurs at 10-25 μM ATP. Mg^{2+} and GTP were significantly less effective than Mn^{2+} and ATP, respectively. When physiologic levels (1mM) of ATP are used, radiolabelled phosphate incorporation is maximal at 250 μM and is observed at the lowest concentration of free Mn^{2+} tested (500nM). Low concentrations of Mn^{2+} ($\leq$2.5μM) remain more effective in promoting the kinase reaction than the higher (2mM) Mg^{2+} concentrations found intracellularly. These findings suggest that the kinase responsible for tyrosyl phosphorylation of the GH receptor in solution is likely to be responsible for its phosphorylation in the intact cell.

F 634 TYROSINE PHOSPHORYLATION AND INTERNALIZATION OF EGF RECEPTORS IN NIH 3T3 CELLS OVEREXPRESSING EGF RECEPTORS. E. Sturani, R. Zippel, L. Morello, R. Brambilla and L. Alberghina. Department of General Physiology and Biochemistry. University of Milan, V. Celoria 26, 20133 Milano, Italy.

The relationships existing between EGF-induced autophosphorylation and dephosphorylation of the receptors, internalization of the ligand-receptor complexes and downregulation of EGF binding sites were investigated in a NIH 3T3 fibroblasts cell line (EGFR T17) which expresses human cloned EGF receptors in the number of 3x105/cell (Velu et al. 1987, Science 238, 1408). Previous data indicated that autophosphorylation of EGF receptors (EGF-R) in Swiss 3T3 and in human fibroblasts was a transient event, while in A431 cells (2x106 receptors/cell) EGF-R remained phosphorylated for a very long time. In addition downregulation of receptors was more complete in fibroblasts than in A431 cells (Sturani et al, Mol. Cell. Biol. 1988, 8, 1345). The kinetics of receptor phosphorylation in EGFR T17 cells was intermediate between that observed in fibroblasts and in A431 cells. In EGFR T17 cells detailed analysis of the fate of a population of receptors previously activated and autophosphorylated showed that dephosphorylation of the receptors and internalization of surface bound 125I-EGF were rapid and followed the same kinetics indicating that the two events took place together. Instead the downregulation of EGF binding sites was slow and only partial. These data suggest that a partial recycling of EGF receptors on the cell surface may take place following internalization of ligand receptor complexes in this cell line.

F 635 MUSCARINIC RECEPTORS AND cGMP, Lily C. Tang, Div. of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC, 20307-5100.

We have demonstrated in vitro that the muscarinic agonists bind to the receptor to open the ion channel. The calcium influx stimulates the guanylate cyclase activity which enhances the cGMP production . This increase in intracellular calcium and cGMP can be abolished by muscarinic antagonists and calcium chelators. A minute increment in calcium concentration can cause the red blood cell (RBC) to lyse. We previously proposed that the function of muscarinic receptor in the RBC is to regulate the intracellular calcium and/or cGMP. We now present the in vivo data in support of this hypothesis. We administered muscarinic agonists alone, or together with muscarinic antagonists or with calcium chelators to male Swiss Albino mice. The red blood cells of the animals treated with muscarinic agonists lysed readily but not the ones co-administered with cholinergic muscarinic antagonists or calcium chelators. The cGMP of the groups treated with oxotremorine increased from 0.334 ± 0.075 ug/mg protein for normal mice to 0.941 ± 0.194 ug/mg protein. The animals that have been given atropine, N-methyl scopolamine, or pirenzepine, their cGMP in the RBC were 0.398 ± 0.097, 0.445 ± 0.13, and 0.390 ± 0.105 ug/mg protein respectively. These results suggest that, in the erythrocyte, the muscarinic receptor-mediated calcium fluxes and cGMP synthesis can be eliminated by the application of a calcium chelator or muscarinic antagonists. Muscarinic receptors may play a role in the regulation of cell membrane rigidity and cell shape.

F 636 TRANSCRIPTIONAL INTERFERENCE BETWEEN ENHANCER FACTORS IDENTIFIES MULTIPLE PATHWAYS TO TRANSCRIPTIONAL ACTIVATION, Diane M. Tasset, Laszlo Tora, Catherine Fromental, and Pierre Chambon, Laboratoire de Génétique Moléculaire des Eucaryotes, 67085 Strasbourg, France

Transcriptional activators are proteins that interact with the transcription machinery and result in productive initiation of transcription by RNA polymerase. A general mechanism for activation of transcription has been described in which relatively short regions of amino acids rich in acidic residues can interact with some component of the transcription machinery. Acidic activation domains (AADs) have been well defined for the yeast trans-activators Gal4 and GCN4, and the herpes simplex virus activator VP16. The steroid hormone family of nuclear receptors are also transcriptional activators that act as enhancer factors when bound to their cognate DNA response element. The activation domains of the human estrogen (hER) and human glucocorticoid receptors (hGR) have been mapped to the N-terminal A/B and C-terminal hormone binding domains (HBDs). The nature of the activation domains of these steroid receptors has been investigated and compared to the AADs of Gal4 and VP16 by a transcriptional interference or squelching assay. In this assay, competition by each activation domain leads to transcriptional interference provided, as for Gal4 and VP16, these activation domains interact with factors important for transcription that are limiting in the cell. Activators interacting with a common factor thus inhibit transcription from activators using a similar mechanism. The results of these experiments suggest that the steroid hormone receptors ER and GR may employ several independent mechanisms of transcriptional activation in addition to, and as well as, that of AADs.

F 637 PHENOTYPIC TRANSFORMATION OF NORMAL RAT KIDNEY CELLS IS UNRELATED TO AUTOCRINE PRODUCTION OF PLATELET-DERIVED GROWTH FACTOR, Everardus J.J. van Zoelen, Dept. of Cell Biology, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands.
Polypeptide growth factors may induce a transformed phenotype onto normal cells, characterized by loss of contact inhibition and induction of anchorage-independent proliferation. In the case of AKR-2B cells growth stimulation by TGFß occurs with a prolonged prereplicative interval, most likely resulting from an indirect growth stimulating mechanism involving induction of the c-sis oncogene and production of a PDGF-like growth factor. In NRK cells TGFß and retinoic acid (RA) are only stimulatory in the additional presence of EGF. Thymidine uptake experiments show that also in this cell line mitogenic stimulation of contact-inhibited cells by TGFß and RA occurs with delayed kinetics. Northern analysis shows that NRK cells constitutively express the genes for various growth factors inclusing PDGF A-chain, bFGF and TGFß, but no c-sis. No enhanced expression was observed in phenotypically transformed cells. Binding studies with radiolabeled PDGF isomers and analysis of conditioned medium show that NRK cells secrete low levels of a PDGF-like growth factor, irrespective of the state of proliferation. It is concluded that phenotypic transformation of NRK cells is not associated with enhanced production of PDGF-like growth factors.

F 638 **M6P/IGFII CI RECEPTOR IN HUMAN BREAST CANCER CELL LINES : CATH D/IGFII BINDING AND PUTATIVE ROLE IN MITOGENESIS.**
Françoise Vignon, Marc Mathieu and Henri Rochefort - INSERM U 148, Unit Hormones et Cancer, 60 Rue de Navacelles, 34090 Montpellier France.
In human breast cancer cells, the lysosomal enzyme Cathepsin D (Cath D) is synthesized and secreted in excess. Both Cath D and IGFII are mitogenic on these malignant cells. We have shown that radiolabeled immunopurified Cath D binds with a high affinity (KD $\sim$ 0.4 nM) to membrane receptor preparations of these cells. The receptor concentration varied among breast cancer cell lines but it was not correlated to steroid hormone receptor presence. By cross-linking studies with 125I-IGFII and 125I-Cath D, we identified that both mitogens share a common 220/270kDa receptor in these cells, on which they bind to two distinct but interacting sites. Mannose-6-phosphate increased IGFII cross-linking which was strongly inhibited (80%) by Cath D. Conversely, IGFII inhibited Cath D cross-linking by 55%. A consequence of these binding interactions was the inhibition of IGFII growth promoting activity by concomitant addition of Cath D in adequate range of concentrations for both ligands. This evidence, in conjunction with the absence of significant inhibition of IGFII mitogenic activity with a monoclonal specific to IGFI receptor (αIR3), strongly suggests that M6P/IGFII CI receptor triggers mitogenic response in these cells, though the second-messenger pathways have not yet been identified.
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F 639 KINASE F_A/GSK-3 AND PHOSPHATASE-1 IN DIFFERENTIATING 3T3-L1 CELLS.
Emma Villa-Moruzzi, Ist. Patologia Generale, Univ. di Pisa, 56100 Pisa, Italy.
In 3T3-L1 cells differentiation to adipocytes is induced in 5 days by dexamethasone, IBMX and IGF-1 and is accompanied by 10-15-fold increase in insulin receptors. In differentiated cells both cytosolic F_A/GSK-3 (the kinase that activates phosphatase-1) and cytosolic phosphatase-1 (Pase-1) are stimulated by insulin. Before differentiation the kinase F_A is mainly membrane-bound and is extracted with Triton X-100/NaCl. The specific activity of the soluble F_A starts to increases within 3 days from cell stimulation to differentiate and attains to ca. 10-fold increase by day 5, while at the same time the membrane-bound F_A decreases 10-15 fold. In differentiated cells the soluble F_A represents over 90% of the total cellular F_A. In non-differentiated cells Pase-1 represents 80-90 % of the cytosolic and membrane-bound Pase and is assayed either as spontaneous activity or as trypsin-stimulated activity (ca. 5-fold higher). Differentiation increases Pase-1 specific activity by ca. 2-fold in both soluble and membrane fraction while the trypsin-stimulated Pase-1 activity increases more in the membranes (ca. 3-4-fold) than in the cytosol (2-2.5-fold). Gel filtration of the same membrane fraction (extracted with Triton X-100/NaCl) shows that a Pase species of 55-60 kDa, which is inactive and detected only after trypsin-treatment, is specifically induced with differentiation.

F 640 HORMONE-STIMULATED PHOSPHOLIPASE A_2 ACTIVATION BY PROTEIN KINASE C IN MDCK CELLS, Barbara A. Weiss, Catherine Godson, and Paul A. Insel, UCSD, Dept. of Pharmacology, La Jolla, CA 92093. Previous work in this laboratory has indicated that hormones may stimulate arachidonic acid and arachidonic acid metabolism (AA) release by multiple pathways that differ in their dependence on protein kinase C (PKC). Specifically, we have shown that α_1-adrenergic-stimulated AA release appears to be more dependent on PKC than bradykinin-stimulated AA release in a clonal isolate from the Madin Darby canine kidney (MDCK) cell line. The current studies were designed to determine the mechanism by which these hormones and PKC regulate AA release. Both epinephrine and bradykinin stimulate phospholipase A_2 (PLA_2) as evidenced by elevated lysophosphotidylethanolamine (LPE) production. This increase in LPE can be blunted by sphingosine, an inhibitor of protein kinase C, and by prior exposure to phorbol esters to down-regulate PKC. Western blot analysis of extracts from phorbol ester down-regulated cells shows that the α isoform of the enzyme is the primary down-regulated, indicating that this isoform may be involved in regulating PLA_2. Incorporation of [^{3}H]arachidonic acid into membrane phospholipids is not effected by either hormone, suggesting that lysophosphatide acyltransferase activity is not regulated by these hormones. Experiments using RHC80267, an inhibitor of diacylglycerol lipase, indicate that this enzyme does not contribute to stimulation of AA release. Thus, we conclude that PKC regulation of PLA_2, perhaps preferentially by the α isoform, is the primary mechanism by which hormones stimulate AA release in MDCK cells.