PHOSPHORYLATION/DEPHOSPHORYLATION IN SIGNAL TRANSDUCTION

Organizers: Mariano Barbacid and Joseph B. Bolen

January 17-24, 1993; Keystone, Colorado

Plenary Sessions *Page*

Poster Sessions

Tyrosine Protein Kinase Receptors

BZ 001 **THE Trk FAMILY OF NEUROTROPHIN RECEPTORS.** M. Barbacid, F.R. Klein, F. Lamballe, S. Jing and P. Tapley. Department of Molecular Biology, Bristol Myers Squibb Pharm. Research Institute, Princeton, New Jersey, 08543.

The NGF family of neurotrophins (NGF, BDNF, NT-3 and NT-4/5) recognize two classes of receptors which can be differentiated by their distinct binding affinities (low and high affinity receptors) and biological properties, since only the high affinity receptors mediate their neurotrophic properties. The low affinity receptors (K_d = 1 nM) have been identified as a single cysteine-rich glycoprotein, $p75^{LNGFR}$, which recognizes each of the above neurotrophins with the same low affinity. The functional, high affinity receptors (K_d = 10 pM) have been recently identified as the members of the Trk family of tyrosine protein kinases. These receptors are responsible for mediating neurotrophin signal transduction through the formation of receptor homodimers in a fashion highly reminiscent of other well characterized tyrosine protein kinase receptors. Co-expression of $p75^{LNGFR}$ with Trk receptors does not have a significant effect on Trk-mediated signal transduction, at least in non-neuronal cells. Moreover, most neurons (particularly in the CNS) that express Trk receptors do not express $p75^{LNGFR}$. These observations suggest that the low affinity $p75^{LNGFR}$ receptors may play an indirect role by facilitating the interaction of these neurotrophins (e.g., by ligand recruitment and/or presentation) with their functional Trk receptors.

To date, we have identified eight Trk receptors encoded by three distinct loci designated *trk*, *trk*B and *trk*C. Whereas the *trk* gene encodes a single tyrosine kinase receptor, $gp140^{trk}$, the related *trk*B and *trk*C genes encode various receptor isoforms. In addition to the canonical tyrosine kinase $gp145^{trk}B$, the *trk*B gene encodes a cell-surface receptor, $gp95^{trk}B$, that lacks the catalytic kinase domain. *trk*C also encodes similar non-catalytic receptors as well as at least three tyrosine kinase isoforms ($gp145^{trk}CK1$, $gp145^{trk}CK2$ and $gp145^{trk}CK3$) which differ in the presence of additional sequences (14 aa. in $gp145^{trk}CK2$ and 25 aa. in $gp145^{trk}CK3$) within their respective kinase domains. Preliminary results indicate that these receptors exhibit different biological properties. Whereas all of them have tyrosine kinase activity and can mediate signal transduction in response to their cognate ligand NT-3, only $gp145^{trk}CK1$ efficiently induces transformation of NIH3T3 cells and neuronal differentiation of PC12 cells.Unlike $p75^{LNGFR}$, the Trk receptors show a high degree of specificity for each of the members of the NGF family of neurotrophins. Whereas NGF exclusively binds to $gp140^{trk}$, the $gp145^{trk}C$ kinase receptor isoforms specifically interact with NT-3. In contrast, $gp145^{trk}B$ can be equally activated by BDNF and NT-4/5. In addition, $gp140^{trk}$ and $gp145^{trk}B$ can be partially activated by NT-3, at least in certain non-neuronal cells. In order to understand the role of the Trk family of receptors in the development and maintenance of the mammalian nervous system, we have targeted the *trk*, *trk*B and *trk*C loci in embryonic stem (ES) cells. So far, we have generated mice carrying a disrupted *trk*B locus (in collaboration with A. Joyner's group at Mount Sinai, Toronto). Preliminary results regarding the phenotype of these *trk*B -/- mice will be presented.

Molecular Interactions and Structures

BZ 002 ACTIVATION OF SERINE/THREONINE KINASES BY TYROSINE PHOSPHORYLATION IN CELLS TRANSFORMED BY THE V-SRC ONCOGENE, Hidesaburo Hanafusa, David Sternberg, and Glen Scholz, Laboratory of Molecular Oncology, The Rockefeller University, New York, NY 10021.

Many serine/threonine protein kinases have been shown to be activated by cell transformation with oncogene tyrosine kinases or growth factor stimulation of receptor tyrosine kinases. Some of these serine/threonine kinases appear to be stimulated by a cascade mechanism in mitogen-stimulated cells: one activated kinase can in turn phosphorylate and activate a second kinase. However, relatively little is known about the initiation of these signaling cascades by activated tyrosine kinases, such as that encoded by the v-src oncogene. Using anti-phosphotyrosine immunoaffinity chromatography, we have searched for serine/threonine kinases that are directly regulated by tyrosine phosphorylation in v-src-transformed rat 3Y1 fibroblasts. Serine/threonine kinase activities that phosphorylated numerous substrates were enriched in v-src-3Y1-transformed cells compared to parental 3Y1 cells. One of these kinase activities, which phosphorylated histone H1 in vitro on serine residues, was further characterized. The histone H1 kinase activity elutes from a MonoQ FPLC column as a single peak, and gel filtration chromatography suggests that the kinase has a molecular mass of approximately 55 kDa. This kinase was inactivated by the LAR tyrosine phosphatase catalytic domain, and this result indicates that tyrosine phosphorylation activates this kinase. Experiments with cells transformed with a temperature sensitive mutant of the v-src oncogene demonstrate that the tyrosine phosphorylation of the H1 kinase is an early event: the H1 kinase was activated (3-4 fold) within 5 min of shift to the permissive temp., and maximally activated (>20 fold) by 30 min. The activity of the kinase remained at this level for greater than 2 hr. When cultures were shifted up to the nonpermissive temp., the activity of the kinase began to decrease within 15 min and returned to the level seen in uninfected cells in 1 hr. The kinase is distinct from known members of the cdc2 and MAP kinase families, and known second messenger-regulated kinases, as assessed by immunological, pharmacological, and biochemical criteria.

The kinase was not activated by c-Src or v-Src(K295M). However, non-myristoylated Src, ΔSH3 mutants, and the weakly transforming mutant, PA104, can stimulate the activity of this serine kinase. The activity of a histone kinase was also found to be elevated in CEF infected with FSV, and to a lesser degree with Y73 and AEV, but not MH2 viruses.

BZ 003 CRYSTAL STRUCTURE OF CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE TO 2.0 Å RESOLUTION AND ITS IMPLICATIONS FOR PROTEIN KINASE FAMILY, DANIEL R. KNIGHTON[1], JIANHUA ZHENG[3], LYNN F. TEN EYCK[3], NGUYEN-HUU XUONG[4], MADHUSUDAN[1], ROLF KARLSSON[1], SUSAN S. TAYLOR[3], and JANUSZ M. SOWADSKI[1], University of California, San Diego, Department of Medicine[1], Biology[2], Chemistry[3] and Physics[4].

Crystal structure of the catalytic subunit of cAMP-dependent protein kinase cAPK complexed with specific peptide inhibitor PKI (5-24) refined to 2.0 Å resolution provide the comprehensive structural information about the homologous catalytic core of the protein-kinase family. This structure along with the structures of MgATP: PKI (5-24): cAPK complex, MgADP: PKI (5-24, Ala 377- Ser) :cAPK complex, and various structures of complexes of bovine heart catalytic subunit provide description of the conformational changes which enzyme undergoes during the catalytic cycle. The homologous catalytic core consists of the conserved unique nucleotide binding motif with five beta-stranded meander located on the upper lobe of the enzyme. The conserved catalytic loop located on the lower lobe of the enzyme provide in particular two conserved ligands (Asn 171, Asp 184) to the magnesium metal doublet. In addition this loop provide essential Lys 168 which is a ligand to the gamma-phosphate of bound MgATP. This essential for catalysis residue is replaced by Arg in the tyrosine-kinase family. Comparison of two structures, one with bound MgADP and peptide inhibitor PKI (5-24) and one with bound MgADP and the substrate peptide (PKI 5-24 Ala 377-Ser) shows substantial movement of the phosphate anchor (glycine rich loop) and the small overall motion of the unique nucleotide binding domain (upper lobe) suggesting that the conformation of the enzyme undergoes the significant changes during the catalysis. This result is also consistent with the preliminary data on the structure of the apo-enzyme (at lower resolution) which shows 10 degrees rotation of the upper domain (nucleotide binding domain) in regards to the lower domain. Fuller structural description of the catalytic cycle requires structures of the complexes of the enzyme with MgADP and phosphorylated PKI (5-24 Ala 377-Ser) and those complexes have been already crystallized. The crystallographic work and the refined model of the catalytic subunit to 2.0Å resolution forms a base for structural modelling studies coupled with the mutagenesis studies and result of this work will be presented in regards to:

1. modelling of the autoinhibitory region of myosin light chain kinase (which resulted in designing the most potent peptide inhibitor)
2. modelling of the tyrosine kinase family with two prime examples EGFR protein kinase and pp60 c-src.
3. modelling of the cdc2 protein kinase and its interactions with cyclins.

Non-Receptors Tyrosine Protein Kinases and Their Substrates

BZ 004 A GENETIC DISECTION OF LYMPHOCYTE SIGNALING, Mark W Appleby, Jane A Gross, Steven D Levin, Michael P Cooke and Roger M Perlmutter. Howard Hughes Medical Institute and Departments of Immunology, Biochemistry and Medicine. University of Washington, Seattle WA98195.

Although the mechanism of signal transduction from the T cell receptor (TCR) remains obscure an increasing body of evidence suggests that two members of the *src* family of protein tyrosine kinases, p56^{lck} and p59^{fyn} are critical components of this signaling process. Both p56^{lck} and p59^{fyn} accumulate specifically in lymphoid cells; in the case of p59^{fyn},two isoforms of the protein, derived as a consequence of the mutually exclusive splicing of alternate seventh exons, have been identified. Transcripts utilizing exon 7B (*fyn*T) accumulate primarily in lymphocytes, whilst exon 7A contributes to *fyn* transcripts in other tissue, notably neurons. A physical association has been described between p59^{fyn}T and the TCR complex, and levels of p59^{fyn}T increase dramatically as thymocytes mature. Experimental manipulation of p59^{fyn} levels in normal T lineage cells, achieved using transgenic model systems, provides direct evidence supporting the involvement of p59^{fyn} in TCR signaling. For example, the overexpression of either isoform of p59^{fyn} under the control of the *lck* proximal promoter results in the production of thymocytes that are acutely sensitive to stimulation through the TCR. Futhermore, mice which lack the thymic isoform of the protein (*fyn*T^{null}), produced via targeted disruption of exon 7B, exhibit a corresponding lymphoid defect: *fyn*T^{null} thymocytes are refractile to stimulation through the TCR with mitogen or antigen. Hence p59^{fyn}T plays a pivotal role in T cell receptor signaling. These animal models permit a partial biochemical dissection of T cell signaling pathways.

BZ 005 SIGNALING BY SRC TYROSINE PROTEIN KINASES IN HEMOPOIETIC CELLS. Joseph B. Bolen, Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey.

The src gene family of tyrosine protein kinases consists of nine known family members: *c-src*, *c-yes*, *fyn*, *lyn*, *lck*, *hck*, *c-fgr*, *blk*, and *yrk*. While the *src*, *yes*, *yrk*, *fyn*, and *lyn* genes are expressed in diverse cell types, the products of the *lck*, *hck*, *fgr*, and *blk* genes are principally expressed in cells of hematopoietic origin. Recent evidence suggests that multiple members of the src family play important roles in signal transduction mediated by a variety of surface receptors in hematopoietic cells. In T lymphocytes Lck and Fyn can be enzymatically activated by engagement of several different T-cell surface proteins including CD4, CD8, CD2, CD28, the T-cell antigen receptor, and the interleukin-2 receptor. In B lymphocytes, cross-linking of the B-cell antigen receptor can activate Blk, Lyn, Fyn, Hck, and Lck. In Mast cells, engagement of the high affinity IgE receptor can activate Lyn, Src, and Yes. These observations suggest that distinct multi-subunit receptors can stimulate many different src family tyrosine kinases through what may be a similar mechanism. Additionally, the signaling pathways of these varied receptors may have common targets that represent substrates of this class of protein kinase.

BZ 006 PROTEIN PHOSPHORYLATION IN LYMPHOCYTE ACTIVATION AND ANTIGEN RECEPTOR GENE REARRANGEMENT, Stephen Desiderio, Susan Dymecki, Weei-Chin Lin, Sami Malek, Janet Siliciano, Craig Smucker, Karen Zeller, Patty Zwollo, Department of Molecular Biology and Genetics and Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

Lymphocytes are activated by interactions with antigens, lymphokines and cell adhesion molecules; tyrosine phosphorylation is implicated in signaling through each of these pathways. To examine functional relationships between individual kinases and specific signal transductory pathways, we have identified tyrosine kinase genes whose expression is restricted to lymphoid cells. One of these, *itk*, specifies a 72-kD tyrosine kinase of unusual structure: while it is related to members of the Src family, it lacks the N-terminal myristoylation consensus sequence and regulatory C-terminal tyrosine residue characteristic of Src kinases. Expression of *itk* is restricted to the T cell lineage, suggesting that the tyrosine kinase encoded by *itk* functions in a T cell-specific signal transduction pathway. Antibodies against peptides specified by *itk* detect a 72 kD phosphoprotein in thymus and T cell lines; as predicted, this protein is associated with protein-tyrosine kinase activity. RNAs for *itk* and for the α chain of the IL-2 receptor are co-induced by the T cell mitogen IL-2, implicating *itk* in the response of T cells to this lymphokine. Activation of B cells by engagement of surface immunoglobulin (sIg) is mediated by tyrosine phosphorylation. We identified a B lymphoid-specific member of the *src* family, *blk*, which encodes a 55-kD protein-tyrosine kinase (p55^{blk}). Expression of *blk* is restricted to the B cell lineage; it is expressed in all pro-B, pre-B and mature B cell lines examined, but is absent from plasma cell lines. In normal mouse spleen, p55^{blk} is restricted to lymphocytes and is concentrated in the follicular mantle, which is rich in sIgM+, sIgD+ B cells; p55^{blk} is not found in plasma cells or stromal cells. The developmental pattern of *blk* expression parallels that of *mb-1* and *B29*, which encode accessory chains of the B cell antigen receptor complex.

Antigen receptors of B and T lymphocytes are encoded in the germline by multiple gene segments that are brought together by site specific DNA rearrangements during development. The products of the Recombination Activator Genes *RAG-1* and *RAG-2* are necessary and sufficient for activation of antigen receptor gene rearrangement. Stability and activity of the RAG-2 protein are apparently regulated by distinct phosphorylation pathways. RAG-1 and RAG-2 are both phosphoproteins. The major sites of RAG-2 phosphorylation in vivo were mapped to two Ser residues within an acidic region. An Ala substitution at one of these sites reduced the ability of RAG-2 to activate antigen receptor gene rearrangement in vivo. Ala substitutions at five other Ser or Thr residues within the acidic domain had no effect on rearrangement. None of these mutations had any effect on expression of RAG-2 RNA or protein. These observations suggest that the activity of RAG-2 is regulated by phosphorylation of a specific Ser residue within the acidic domain. The RAG-2 sequence contains several potential p34^{cdc2} phosphorylation sites; two of these are selectively phosphorylated by p34^{cdc2} or a related kinase in vitro. Substitution of Ala for Thr at the major p34^{cdc2} phosphorylation site resulted in a large increase in the steady state level of RAG-2 protein. The relative increase in RAG-2 expression is most likely the result of protein stabilization, because (1) mutant and wild type RNA levels were identical and (2) chimeric proteins containing wild type or mutant RAG-2 polypeptide segments exhibited a similar difference in steady state level. These observations suggest that phosphorylation of RAG-2 by p34^{cdc2} or a related kinase increases its rate of degradation. By such a mechanism, antigen receptor gene rearrangement may be coupled to the cell cycle.

BZ 007 FOCAL ADHESION KINASE, SRC FAMILY KINASES AND CELLULAR SIGNALLING, Michael Schaller, Jeffery Hildebrand, Tzeng-Horng Leu, and J. Thomas Parsons, Department of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA.

Protein tyrosine kinases play a pivotal role in the regulation of a wide variety of cellular growth signals. Expression of activated protein tyrosine kinases, such as oncogenic forms of $pp60^{src}$, leads to the increase in tyrosine phosphorylation of numerous cellular proteins, alterations in cell morphology and perturbation of growth control mechanisms. We have isolated monoclonal antibodies directed to 7 distinct phosphotyrosine containing proteins from Rous sarcoma virus transformed chicken embryo cells (1). One of these antibodies recognizes a 125 kDa protein present in cellular focal adhesions. Focal adhesions define regions of close contact between cells and the substrate upon which they grow and contain cell surface receptors for extracellular matrix proteins (integrins), as well as numerous cytoskeletal proteins (talin, vinculin, tensin, paxillin). Sequence analysis of cDNAs encoding pp125 revealed that pp125 is a protein tyrosine kinase, which we have termed FAK, for Focal Adhesion Kinase (2). $pp125^{FAK}$ encodes a unique kinase which is characterized by a highly conserved tyrosine kinase domain flanked by a large (approximately 380 amino acid) C-terminal domain and a 390 residue amino terminal domain, devoid of SH2 and SH3 motifs. In avian cells, alternative splicing of FAK RNA yields two mRNAs, a 4.8 kilobase mRNA that encodes $pp125^{FAK}$, and a shorter mRNA that encodes a 41 kDa protein (referred to as FRNK or FAK-Related Non Kinase). $p41^{FRNK}$ is identical in sequence to the C-terminal 41 kDa of $pp125^{FAK}$. Engagement of integrins with extracellular ligands, including the spreading of embryo cells on fibronectin, causes a 2 to 4 fold increase in $pp125^{FAK}$ tyrosine phosphorylation and an increase in its enzymatic activity, measured in vitro. In addition, fibronectin mediated spreading of CE cells is accompanied by the serine/threonine phosphorylation of $p41^{FRNK}$, suggesting the coupled modification of $pp125^{FAK}$ on both tyrosine and serine/threonine. A structure-functional analysis of $pp125^{FAK}$ as well as the autonomous expression of FRNK, indicates that association of FAK and FRNK with focal adhesions is mediated by the C-terminal domain and may be regulated by serine/threonine phosphorylation. In *src* transformed CE cells the tyrosine phosphorylation of $pp125^{FAK}$ is significantly elevated. Greater than 80% of $pp125^{FAK}$ becomes stably associated with $pp60^{src}$. The near quantitative association of $pp125^{FAK}$ with $pp60^{src}$ indicates that complex formation may contribute to the transformed phenotype. This is further supported by the observation that $pp125^{FAK}$ does not associate with transformation defective src variants bearing mutations within the SH2 domain. Identification of pp125 as an integrin stimulated, focal adhesion kinase suggests that $pp125^{FAK}$ activation may play a direct role in regulating assembly/disassembly of cytoskeletal components of focal contacts, possibly by the direct phosphorylation of focal adhesion components. Alternatively, activation of $pp125^{FAK}$ may signal the translocation of $pp60^{src}$ or other *src* family kinases to the focal adhesion, thus coupling the activation of FAK to the phosphorylation of focal adhesion components by other tyrosine kinases and/or phosphorylation of more conventional components of a signal transduction pathway.

(1) Kanner et al., (1990) Proc. Natl. Acad. Sci. USA, 87:3328-3333.
(2) Schaller et al., (1992) Proc. Natl. Acad. Sci. USA, 89:5192-5196.

Cell Cycle-I (Joint)

BZ 008 CELL CYCLE PHOSPHORYLATION, Tony Hunter, Jeroen den Hertog, Rick Lindberg, Jill Meisenhelder, David Middlemas, John Pines, Byron Sebastian, Sharon Tracy, and Peter van der Geer, The Salk Institute, La Jolla, California 92037

Receptor protein-tyrosine kinases (PTKs) transduce signals across the plasma membrane in response to extracellular ligands. Many receptor PTKs act to trigger cells to enter the cell cycle from the G0 state. CSF-1 is a growth factor for myeloid precursors, and the CSF-1 receptor is a PTK in the PDGF receptor PTK family. We have identified Tyr697, 706, and 721 (the site responsible for the binding of PI-3 kinase) in the CSF-1 PTK kinase insert as autophosphorylation sites, and analyzed their role in CSF-1-mediated immediate early gene induction, growth stimulation, and morphological responses by mutagenesis. Eck is a receptor-like PTK , which is mainly expressed in epithelial cells, and which is a member of a family of receptor PTKs, (Eph, Elk, Eek, Cek4/Mek4, Sek, Cek5/Nuk). We have identified a potential ligand for Eck in conditioned media of transformed cells. The TrkB receptor PTK is closely related to but distinct from the Trk and TrkC receptor PTKs. *trk*B is primarily expressed in brain as a 140 kDa glycoprotein and two smaller ~90 kDa C-terminally truncated proteins. TrkB PTK activity is stimulated by NT-3 and BDNF. NT-3/BDNF stimulates phosphorylation of TrkB on two Tyr, and phosphorylation of PLCγ1, and its association with activated TrkB. In collaboration with Hakan Persson (Karolinska Institutet) we have found that kindling-induced hippocampal seizures cause rapid but transient inductions of *trk*B mRNAs and TrkB proteins in rat hippocampus, without any effect on the levels of *trk* and *trk*C RNAs. Concomitantly BDNF mRNA is induced in the same hippocampal areas, suggesting that paracrine or autocrine stimulation of TrkB is involved in repair of neuronal damage. To study the role of protein-tyrosine phosphatases (PTPs) in the cell cycle we have cloned the mouse homologue of the PTPα receptor-like PTP. PTPα is phosphorylated constitutively in NIH3T3 cells, predominantly on two Ser, which we have identified as Ser180 and Ser204. TPA treatment of NIH3T3 cells stimulates phosphorylation at both Ser, and recombinant PTPα is phosphorylated at Ser180 and Ser204 by purified protein kinase C. Phosphorylation of PTPα at Ser180 and Ser204 increases its PTP activity in vitro. We have identified the SH3/SH2 domain-containing protein Nck as a novel substrate for the PDGF and EGF receptor PTKs, and the vSrc oncoprotein.

Progression through the somatic cell cycle is governed by a series of cyclin/cdk complexes that are formed and activated at different times during the cycle. The G2/M transition is controlled by cyclin A/cdc2 and cyclin B1/cdc2 complexes, that are activated by action of the cdc25 phosphatase on cdc2. We have proved that cdc25B can act as a dual specificity protein phosphatase, which can dephosphorylate both P.Thr14 and P.Tyr15 in cyclin B1-associated cdc2. Cyclin B1 is accumulates in the cytoplasm until the start of prophase, and is then translocated into the nucleus before nuclear lamina breakdown. In prophase cyclin B1 localizes to the mitotic asters, and in metaphase cyclin B1 localizes to the spindle poles and microtubules. In contrast to cyclin B, cyclin A is predominantly a nuclear protein, and is associated with cdk2 rather than cdc2. We have analyzed cyclin A/B1 chimeras to determine what dictates the localization of the two cyclins and the association with different cdks. Unexpectedly we have found that cyclin A/cdk2 complexes are stimulated by cdc25B treatment, implying that this complex is negatively regulated by phosphorylation, and we have identified Thr14 and Tyr15 in cdk2 as the regulatory phosphorylation sites. We have also found that cyclin E/cdk2 complexes are also activated by cdc25B. This implies that different cdc25's may play a role in regulating transitions in the cell cycle other than G2/M.

BZ 009 GROWTH FACTOR-REGULATED MAMMALIAN G1 (D-TYPE) CYCLINS AND THEIR CYCLIN-DEPENDENT KINASES. Charles J. Sherr,[1,2] Martine F. Roussel,[2] David Strom,[2] Jun-ya Kato,[2] and Hitoshi Matsushime,[1,2] Howard Hughes Medical Institute and Department of Tumor Cell Biology, St. Jude Children's Research Hospital, Memphis, TN 38105.

Colony-stimulating factor-1 (CSF-1) is required throughout the G1 phase of the macrophage cell cycle in order for cells to enter S phase, after which they can complete the S, G2, and M phases in its absence. Macrophages deprived of CSF-1 accumulate in early G1 and progress synchronously through the cell cycle after readdition of the growth factor, but withdrawal of CSF-1 at any time during G1 results in their inability to enter S phase. Different D-type cyclins (originally designated *CYL* genes) are expressed in G1 as part of the delayed early response to growth factor stimulation. The three murine cyclin D genes are much more related to their human counterparts than to each other, suggesting nonredundancy of their functions. In CSF-1-starved macrophages restimulated to enter the cell cycle, cyclin D1 synthesis is maximally induced in early G1 and persists as long as CSF-1 is present; much lower levels of cyclin D2 accumulate maximally at the G1/S transition, whereas cyclin D3 is not expressed in these cells. Different patterns of cyclin D expression are observed in other proliferating cell types, depending on their lineage.

The cyclin D1 encoded polypeptide, $p36^{D1}$, forms complexes during G1 with a novel cyclin-dependent, serine/threonine kinase ($p34^{cdk4}$) distinct from the known cdk's, $p34^{cdc2}$ (*cdk*1), $p33^{cdk2}$, and $p36^{cdk3}$. When CSF-1-deprived macrophages are stimulated to enter the cell cycle, $p34^{cdk4}/p36^{D1}$ complexes accumulate during G1 and then decrease during S phase, in concert with a rise and fall in *cdk*4 mRNA levels. By late G1, the majority of $p34^{cdk4}$ is found in complexes, with free $p36^{D1}$ present in excess. Thus, expression of cyclin D1 in macrophages is growth factor-regulated and nonperiodic, whereas *cdk*4 gene expression appears to be cell cycle-regulated and, possibly, rate limiting for complex formation. Cyclins D2 and D3 form complexes with $p34^{cdk4}$ in interleukin-2-dependent T cells. We do not exclude the possibility that D-type cyclins might also interact with other *cdk* partners.

In collaboration with Mark E. Ewen and David M. Livingston (Dana Farber Cancer Institute, Boston, MA), we found that D-type cyclins can form stable complexes *in vitro* with the retinoblastoma gene product, pRb. Binding of pRb to $p35^{D2}$ or $p34^{D3}$ requires amino acid sequences within the pRb "pocket" that are necessary for its interactions with DNA tumor virus transforming proteins (T antigen, E1a, and E7), as well as the intact pRb C-terminus. Because cyclins D2 and D3 preferentially associate *in vitro* with pRb, whereas cyclins D1 and D3 are the most efficient in forming *in vitro* complexes with $p34^{cdk4}$, stable ternary complexes containing pRb, cyclin D3 and $p34^{cdk4}$ can be readily assembled from recombinant proteins. Although $p34^{cdk4}$ alone lacks kinase activity, such complexes exhibit a serine/threonine kinase activity that phosphorylates pRb, but not exogenously added, soluble histone H1. Neither of two biologically inactive pRb mutants (Cys-706 to Phe or a C-terminal truncation mutant) assembled into complexes with cyclin $D3/p34^{cdk4}$ or underwent phosphorylation *in vitro*. In contrast, a kinase-defective $p34^{cdk4}$ mutant (containing Met for Lys at its ATP binding site) formed ternary complexes with cyclin D3 and pRb but was unable to catalyze pRb phosphorylation. Cotransfection of pRb together with cyclins D2 or D3 into Rb-negative SAOS-2 osteosarcoma cells induced pRb hyperphosphorylation, suggesting that D-type cyclin-regulated kinase(s) might, at least in part, regulate G1 exit through this mechanism.

BZ 010 Mos PROTO-ONCOGENE AND CELL CYCLE REGULATION. George F. Vande Woude, Renping Zhou, Ira Daar, Nelson Yew, Wayne Matten, Kenji Fukasawa, B.K. Sathyanarayana, and J. Ronald Rubin. ABL-Basic Research Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702.

The mos proto-oncogene product, $p39^{mos}$, is required for meiotic maturation in vertebrate oocytes and is an active component of cytostatic factor (CSF), an activity in unfertilized amphibian eggs believed to be responsible for their arrest at metaphase II. CSF arrests egg development at M-phase and is believed to function by stabilizing maturation promoting factor (MPF). Thus, $p39^{mos}$ functions downstream in the signal pathway during M-phase, and at a major cell cycle control point. Its activity is indirectly responsible for the stabilization of MPF at metaphase II, but we have also shown that Mos is necessary and sufficient to initiate G2/M transition. This link between proto-oncogene function and M-phase cell cycle regulation could be responsible for certain phenotypes of transformed cells. Thus, we have shown that the ras oncoprotein can also substitute for Mos in meiotic maturation and has authentic CSF activity.

We have found that $p39^{mos}$ is associated with and phosphorylates tubulin in vitro. Our analyses have also shown that β-tubulin is preferentially associated with and phosphorylated by $p39^{mos}$ from either transformed cells or unfertilized eggs. In transformed cells, $p39^{mos}$ co-localizes with microtubules including those of the metaphase spindle pole and early telophase mid-body and asters. Moreover, we have determined that $p39^{mos}$ binds to the tubulin heterodimer with a Kd in the nanomolar range, and have identified regions on $p39^{mos}$ which associate with tubulin that correspond to the putative substrate binding region in a 3D model of the Mos kinase derived from the cAMP-dependent protein kinase (cAPK) crystal structure. We speculate that Mos may contribute to the formation of the spindle pole as well as the spindle and thereby contribute, as CSF, to metaphase arrest. Constitutive expression of $p39^{mos}$ in somatic cells is sufficient for morphological transformation, but only cells expressing low levels of Mos can grow as transformed cells. We postulate that this amount of product is not sufficient to cause mitotic arrest but is sufficient to impart M-phase phenotypes during interphase. Altered cell morphology and loss of contact inhibition during interphase could be due to cytoskeletal changes that normally occur during M-phase. Overexpression of Mos in somatic cells leads to growth arrest, inappropriate chromosome condensation and karyokinesis in the absence of cytokinesis. The latter process is perhaps related to the regulatory role of Mos in nuclear division during meiosis. It can also provide an explanation for chromosomal instability in tumor cells.

Research supported by the National Cancer Institute, DHHS, under contract No. NO1-CO-74101 with ABL.

Cell Cycle-II (Joint)

BZ 011 KINASES AND PHOSPHATASES THAT REGULATE THE CELL CYCLE IN DROSOPHILA. David M. Glover, Cancer Research Campaign Laboratories, Cell Cycle Genetics Group, Department of Anatomy and Physiology, Medical Sciences Institute, The University, Dundee DD1 4HN, Scotland, UK.

The entry into mitosis is universally regulated by the $p34^{cdc2}$ kinase, which is itself activated by the tyrosine phosphatase cdc25 or its homologues. Drosophila has two cdc25 homologues, encoded by the genes string and twine, which we have isolated by their ability to rescue a fission yeast ts mutant. Whereas string appears to control entry into mitosis in somatic tissue, twine is expressed exclusively in the germ-line. Meiosis does not occur in homozygous twine males which produce cysts containing 16 rather than 64 spermatids, and in homozygous twine females, it occurs prematurely.

Both cdc2 and cdc25 homologues appear to be regulated by their phosphorylation state. Both serine-threonine protein phosphatases PP1 and PP2A have been implicated in this control. Drosophila mutants in the gene for the major isoform of PP1 and 87B show an elevated mitotic index and have cells with overcondensed chromosomes, and collapsed or multipolar spindles. Mutations in the gene for the 55 kDa regulatory subunit of PP2A at 85F, on the other hand, show two specific types of anaphase defect. Most frequently we observe intact lagging chromatids that have undergone separation from their sisters, but which remain at the position formerly occupied by the metaphase plate. This is suggestive of a specific anaphase function. The second class of abnormal anaphase figures show stretched chromosomes in which chromatids remain attached, but not at their centromeric regions, so as to bridge the mitotic poles, possibly reflecting a defect earlier in S-phase.

Relatively little is known of the phosphatases that oppose kinases other than cdc2 required for the mitotic cycle. aurora, a Drosophila gene essential for centrosome separation, encodes a 40 kDa serine-threonine protein kinase. Mutation in aurora results in monopolar spindles in larval neuroblasts. Whereas the function of aurora seems to be required early in the mitotic cycle, mutation in polo leads to a variety of abnormal late events in mitosis. These include bipolar spindles in which one pole can be unusually broad; and monopolar spindles, as well as non-disjunction in meiosis. polo encodes a protein kinase that is the Drosophila homologue of that encoded by the budding yeast gene cdc5. Assays for polo kinase activity in the mitotic cycles of syncytial embryos shows it to have cyclical activity peaking at late anaphase-telophase.

BZ 012 BIOCHEMICAL ANALYSIS OF SIGNALS COMING TO AND EMITTED BY RB AND OTHER CELL CYCLE REGULATING POCKET PROTEINS. D. M. Livingston, T. Chittenden, J. DeCaprio, R. Eckner, M. E. Ewen, P. Farnham*, Y. Li*, E. Flemington, W. Kaelin, W. Krek, N. Modjtahedi, C. Sherr**, and S. Shirodkar. Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA; *McArdle Laboratory, University of Wisconsin, Madison WI; and, **St. Jude Hospital and Research Institute, Memphis, TN.

RB, p107, and p300 are all specific nuclear binding targets of E1A and SV40 T antigen, both DNA tumor viral transforming proteins. Binding by the latter to these proteins is believed to contribute measurably to their transforming function. Indeed, there is abundant evidence that each of these proteins can operate in one or more aspects of cell cycle control. In the cases of RB and p107, cell cycle regulation is provided, at least in part, by each of these proteins modulating, in a timely manner, the action of at least one transcription factor, E2F-1, which, itself, is believed to promote exit from G0/G1 and passage through S. E2F-1 has now been cloned and aspects of its structure suggest possible ways in which RB and p107 might influence its action.

In its un(der)phosphorylated state, RB is believed to contribute to a block to exit from G0/G1 which can be overcome, at least in part, by specific RB phosphorylation beginning in the second half of G1. Recently, we have accumulated evidence suggesting a possible role of one or two D-type G1 cyclin-cdk kinase complexes in the process of releasing cells from a G1 block erected by un(der)phosphorylated RB. Specific interactions between RB and each of these two cyclins were detected in vitro. In addition, we have accumulated indirect evidence pointing to analogous interactions in vivo. The data imply that one or more D cyclin-cdk complexes interact, directly or indirectly, with unphosphorylated RB in vivo and that at least one outcome of these encounters is passage out of G1.

Serine/Threonine Kinases

BZ 013 SIGNALING THROUGH TGF-ß RECEPTORS. Joan Massagué, Liliana Attisano, Juan Cárcamo, Fernando López-Casillas, Jacqueline Doody, Jeffrey L. Wrana and Alejandro Zentella. Howard Hughes Medical Institute and Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Transforming growth factor-ß (TGF-ß) belongs to a large family of secretory polypeptides that are broadly multifunctional as regulators of cell growth, differentiation and tissue organization, expressed in many cell types, and highly conserved in evolution. Cell interaction with the three mammalian isoforms, TGF-ß1, ß2 and ß3, involves various membrane proteins including the ubiquitous high affinity receptors I and II, two type III receptors (betaglycan and endoglin), and two glycolipid-anchored binding proteins that are specific for different TGF-ß isoforms. Betaglycan is a membrane-anchored proteoglycan that binds TGF-ß1, ß2 and ß3 through its core protein. Betaglycan is structurally related to endoglin, a membrane glycoprotein of endothelial cells that binds TGF-ß1 and ß3 but not TGF-ß2.

Betaglycan presents TGF-ß receptor II. The TGF-ß receptor II belongs to a family of membrane serine/threonine protein kinases. Receptor II signals TGF-ß responses through its kinase in association with receptor I. Receptors I and II are interdependent components of a heteromeric receptor complex: receptor I requires receptor II to bind TGF-ß, and receptor II requires receptor I to signal. This mode of operation points to fundamental differences between this receptor and the tyrosine protein kinase cytokine receptors.

BZ 014 TRACING SIGNALS FROM THE MEMBRANE TO THE NUCLEUS, Sadhana Agarwal, Nidhi Williams, Ken Wood, Wayne Haser, Kurt Auger, Ana Carrera, Fred King, Katherine Campbell, Brian Druker, David Pallas and Tom Roberts, Dana Farber Cancer Institute and Harvard Medical School, Boston, MA

The study of signal transduction from receptors at the plasma membrane to the transcription apparatus in the cell's nucleus requires a variety of systems designed to assay particular parts of the process. Several such systems will be described. The combination of a synthetic gene for a tyrosine kinase with an efficient expression and purification system is used to study structure/function relationships in the initiating tyrosine kinases. A PC 12 cell system has been constructed in which a series of clones allow the inducible expression both the activated and dominant interfering forms of a variety of signal transducers. Since PC12 cells, unlike fibroblasts, survive when key mitogenic signal transducers, such as ras and raf, are knocked out, this system is particularly useful to observe the requirements for particular elements in a given signaling step. Finally the baculovirus system allows the expression of multiple signal transducing elements in a system which facilitates genetic controls. The infected insect cells become living test tubes to study interactions between transducing elements. This system has been particularly useful in studying the interaction of kinases and in elucidating the role of given kinases in the activation of transcription factors. Recent results from these and other systems will be described.

BZ 015 MAP KINASE AND MAP KINASE KINASE: REGULATION BY TYROSINE AND THREONINE PHOSPHORYLATIONS, Michael J. Weber[1], Jeng-Horng Her[1], Saquib Lakhani[1], Mukund Nori[1], Gilles L'Allemain[1], Jordi Vila[1], Jie Wu[2], Paul Dent[2] and Thomas W. Sturgill[2], [1]Department of Microbiology and [2]Pharmacology and Medicine, University of Virginia Health Sciences Center, Charlottesville, VA 22908.

MAP kinases are serine/threonine specific protein kinases which are regulated by dual tyrosine and threonine phosphorylation in response to stimulation of cells with diverse agonists. Two pathways for MAP kinase activation have been delineated, one dependent on $p21^{ras}$, and the other dependent on heterotrimeric G proteins. In somatic cells, these enzymes are likely to play an important role in $G_0/G1$ transitions involving growth or differentiation. Phosphorylation on both tyrosine and threonine is necessary for full enzymatic activity.

Although these kinases function as serine/threonine kinases, they are able to autophosphorylate on tyrosine. The site of tyrosine autophosphorylation is the same site which is phosphorylated following agonist stimulation of cells. Although this raises the possibility that autophosphorylation could play a role in the *in vivo* activation of MAP kinase, no "autokinase enhancing factor" has been found. Rather, the *in vivo* activation of MAP kinase can be accounted for by a "MAP kinase kinase" which catalyzes both the threonine and tyrosine phosphorylations. Thus, MAP kinase is a serine/threonine specific protein kinase for exogenous substrates, but autophosphorylates on tyrosine. However, the MAP kinase kinase is a dual-specificity kinase for its exogenous substrate.

Using purified MAP kinase kinase and recombinant mutant forms of MAP kinase substrates, we found that the phosphorylations of MAP kinase on threonine or tyrosine can occur independently of each other, and can occur with a MAP kinase mutant defective in ATP binding. The requirements for function and activation of MAP kinase kinase are being further analyzed.

Second Messengers

BZ 016 ROLE OF SH2 DOMAINS IN SIGNAL TRANSDUCTION: PI 3-KINASE AND OTHER SIGNAL TRANSDUCERS, Lewis Cantley, Dept. of Physiology, Tufts University School of Medicine, Boston MA 02111.

Signal transduction by activated protein-tyrosine kinases often involves formation of tight complexes between proteins that are phosphorylated on tyrosine and a second group of proteins that have src homology 2 (SH2) domains. The biochemical basis for this complex formation is now known to involve direct binding of the phosphotyrosine moiety in a highly conserved region of the SH2 domain. The amino acids C-terminal to the phosphotyrosine residue of the bound peptide provide sequence specificity to this interaction by interacting with less-conserved residues on the SH2 domain. We have developed a technique using a library of degenerate phosphopeptides to determine the optimal phosphopeptide for binding to individual SH2 domains. Using this technique we have shown that the optimal peptide for binding to the N-SH2 domain of the p85 subunit of PI 3-kinase is PhosphoTyr-Met/Val-X-Met (where X is any amino acid). The optimal peptide for the C-SH2 of p85 is PhosphoTyr-X-X-Met. These sequences are consistent with the known binding sites for PI 3-kinase in polyoma middle t, the PDGF receptor, CSF-1 receptor and Steel receptor. The optimal binding sequence for the src SH2 domain is PhosphoTyr-Glu-Glu-Ile. This sequence is found in an interesting region of the Hamster polyoma middle t protein. A synthetic phosphopeptide based on the region of Hamster middle t containing this sequence binds to the src SH2 domain with a Kd of about 40 nM. The PhosphoTyr-Glu-Glu-Ile peptide is predicted to fit tightly into the src SH2 crystal structure published by Waksman et al., Nature 358, 646-653 (1992). The two glutamate residues are predicted to bind to Lys and Arg residues of the central beta strand and the Ile is predicted to fit into a hydrophobic pocket. Using the degenerate phosphopeptide library we have determined the optimal peptide sequences for binding to 10 additional SH2 domains. For each SH2 domain-phosphopeptide complex, the amino acids selected at the +1, +2 and +3 positions C-terminal to the PhosphoTyr can be rationalized with the side chains of the SH2 domains at the predicted sites of contact (based on alignment with the src SH2 crystal structure). These motifs predict likely binding sites for signal transduction molecules on growth factor receptors and other signal transduction molecules. Thus, signaling complexes may be predicted on the basis of primary structure.

BZ 017 REGULATION OF RAS FUNCTION BY GUANINE-NUCLEOTIDE RELEASING FACTORS, Larry A. Feig, Department of Biochemistry, Tufts University School of Medicine, Boston, MA

The stimulation of receptor protein-tyrosine kinases promotes the active, GTP-bound form of Ras proteins. This is a critical step in signal transduction since inhibition of Ras function by Ras antibodies or dominant inhibitory Ras mutants blocks many of the effects of these receptors on cellular function. In order to reach the active GTP-bound state, Ras proteins must first release bound GDP. This rate-limiting step in GTP binding is catalyzed by guanine-nucleotide releasing factors (GRFs). Deactivated occurs by hydrolysis of GTP to GDP by Ras, and this reaction is catalyzed by GTPase activating proteins (GAPs). Whereas much information has been obtained about GAP proteins, GRFs have only recently been cloned from mammalian systems.

We have recently cloned cDNAs from a rat brain library that encode a ~ 140 kDa GRF for Ras p21 ($p140^{Ras\text{-}GRF}$). Its C-terminal region, which is similar to that of CDC25, a GRF for S. cerevisiae RAS, accelerated the release of GDP from RasH and RasN p21 in vitro, but not from the related RalA, or CDC42Hs GTP-binding proteins. Interestingly, a region in the amino-terminal end of Ras-GRF is similar to the *dbl* oncogene product, a guanine-nucleotide releasing factor for the Ras-related CDC42 protein. CDC42 is a member of the Rho family of GTPases that have been implicated in the regulation of the cytoskeleton. This suggests that $p140^{Ras\text{-}GRF}$ is a dual function releasing factor for Ras and Rho-like proteins.

Expression of $p140^{Ras\text{-}GRF}$ was detected only in brain. Since Ras proteins in all cells presumably require GRFs for activation, a family of Ras-GRFs likely exist. In fact, a different putative Ras-GRF, the mammalian homologue of the drosophila SOS gene, has recently been cloned and shown to be expressed ubiquitously.

Properties of $p140^{Ras\text{-}GRF}$ will compared to those of the ubiquitous releasing factor SOS. Discussion will include how unique features of $p140^{Ras\text{-}GRF}$ might contribute to brain-specific Ras function.

Phosphorylation of Transcription Factors-I (Joint)

BZ 018 MYC IS INVOLVED IN A NETWORK OF PROTEIN INTERACTIONS, D. Ayer[1], E.M. Blackwood[1,2], M.W. King[3], L. Kretzner[1], K. Tietje[1], and R.N. Eisenman[1]. [1]Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle; [2]Department of Pathology, University of Washington School of Medicine, Seattle; [3]Center for Medical Education, Indiana State University, Terre Haute.

The proteins encoded by the *myc* family of proto-oncogenes are members of a larger group of proteins distinguished by the presence of a basic region followed by putative helix-loop-helix and zipper domains (bHLH-Zip proteins). This region is known to mediate DNA binding and protein-protein interactions. Myc family proteins self-associate and bind DNA poorly but readily form sequence-specific DNA binding complexes with Max, another bHLH-Zip protein. Max is a highly conserved protein that can form homodimers but preferentially heterodimerizes with Myc. *In vitro*, Max homodimers bind the CACGTG E-box consensus sequence less tightly than Myc:Max homodimers. In avian and mammalian cells alternative splicing generates two Max proteins (Max and Max9) which differ by a nine residue insertion N-terminal to the basic region. Max proteins in *Xenopus laevis* differ from human Max by the absence of a 24 residue region and from each other by a 27 residue insertion, both in the region C-terminal of the HLH-Zip. In gel retardation assays both human Max9 homodimers and Max9:Myc heterodimers display a significantly slower off-rate for binding to CACGTG than the corresponding complexes with Max.

In vivo, Max proteins have been found to be highly stable and expressed at essentially equivalent levels in resting and proliferating cells. Since Myc is so highly regulated and its short half-life is not affected by heterodimer formation we presume that *in vivo* the ratio of Max homodimers to Myc:Max heterodimers is continuously dependent on the rate of synthesis of Myc.

Since Max is present at times when Myc is not expressed and is generally in excess when Myc is expressed we were led to search for other proteins that might interact with Max. Using Max as a probe we screened a λgt11 expression library and identified a novel bHLH-Zip protein: Mad. Mad homodimerizes poorly but binds Max *in vitro* to form a sequence-specific DNA binding complex with properties very similar to those of Myc:Max. Both Myc:Max and Mad:Max heterocomplexes are favored over Max homodimers and have similar binding specificity and apparent stability. Furthermore, unlike Max, the DNA binding activity of the heterodimers is unaffected by CKII phosphorylation. Mad does not associate with Myc nor with other bHLH-Zip proteins tested.

To determine the potential role of these proteins in gene expression we carried out *in vivo* transactivation assays using a reporter gene linked to promoter-proximal CACGTG binding sites. These experiments demonstrate that Myc:Max complexes activate, while Max homodimers and Mad:Max complexes repress, transcription. Our findings suggest that Max lies at the center of a network of interacting proteins in which the relative levels of Max's dimerization partners can generate opposing transcriptional functions. Since Myc's function appears to be related to proliferation and differentiation, we believe this network may regulate genes that control these aspects of cell behavior.

BZ 019 SIGNAL TRANSDUCTION BY TYROSINE PHOSPHORYLATION OF TRANSCRIPTION FACTORS: A DIRECTOR EFFECTOR MODEL Xin-Yuan Fu and Guang-Rong Sun, Department of Biochemistry, Mount Sinai School of Medicine, New York, NY 10029

The primary transcription factor induced by interferon-α (termed ISGF3, interferon Stimulated gene factor 3) is a complex of four (113, 91/84 and 48 kD) proteins. The 113 and 91/84 kD proteins are present in the cytoplasm before interferon treatment and translocated to the nucleus in response to interferon-α. Sequence comparison has shown that the 113 and 91/84 kD proteins have 42% sequence identities but are derived from different members of a new gene family.

It has been shown that the 113 and 91/ 84 kD proteins of ISGF3 contain conserved SH2 and SH3 (*src* homology regions 2 and 3) domains and are immediately phosphorylated in the cytoplasm by an interferon-α-induced protein tyrosine kinase. Phosphorylated 113 and 91/84 kD proteins can be immunoprecipitated by specific anti-phospho-tyrosine antibodies as well as antibodies against proteins of ISGF3. A phospho-amino acid analysis of [^{32}P]-labeled 113 and 91 kD proteins has shown predominant tyrosine phosphorylation of both proteins only after interferon-α treatment. Moreover, a tyrosine kinase activity associated with the ISGF3 complex is detected in an *in vitro* kinase assay.

Two kinase inhibitors, staurosporine and genistein, inhibit this interferon-α-induced tyrosine phosphorylation of the 113 and 91/84 kD proteins both *in vivo* and *in vitro*. The tyrosine phosphorylated 113 and 91/84 kD proteins can form active ISGF3 complexes with the purified 48 kD protein *in vitro* to bind an interferon stimulated regulatory element (ISRE). Phosphatase treatment of these 113 and 91/84 kD proteins results in inhibition of this ISGF3 complex formation *in vitro*. These observations indicate that interferon-a-induced tyrosine phosphorylation is necessary for activation of the transcription factor ISGF3 and expression of induced genes. A "DIRECT EFFECTOR" model has been proposed on the mechanism of signal transduction induced by interferon.

We are in progress of further identifying the tyrosine kinase involved in this direct signaling, and dissecting functional domains of ISGF3 proteins which may interact with the tentative kinase.

BZ 020 POSITIVE AND NEGATIVE REGULATION OF AP-1 AND OTHER TRANSCRIPTION FACTORS BY PROTEIN PHOSPHORYLATION CASCADES, Michael Karin, Tod Smeal, Bernard Binetruy, Tiliang Deng and Anning Lin, Department of Pharmacology, School of Medicine, University of California, San Diego, La Jolla, CA 92093-0636.

AP-1 is a transcriptional activator composed of homo- and heterodimeric Jun and Jun/Fos complexes. It is involved in the activation of various target genes, such as: collagenase, stromelysin, IL2 and TGFß1, by tumor promoters, growth factors and cytokines. In addition, AP-1 activity is elevated in response to expression of transforming oncogenes including *H-ras*, *v-src*, and *v-raf* and is required for cell proliferation. AP-1 activity is subject to complex regulation both transcriptionally and post-transcriptionally. Transcriptional control determines which of the *jun* and *fos* genes is expressed at any given time in any given cell type. Therefore, transcriptional control determines the amount and composition of the AP-1 complex. Transcription of the *jun* and *fos* genes is subject to both positive and negative autoregulation and is highly inducible in response to various stimuli including those associated with cell proliferation. AP-1 activity is also regulated at the post-transcriptional level. Both cJun and cFos are phosphoproteins that are subject to regulated phosphorylation. In the case of cJun, phosphorylation of sites near the DNA-binding domain inhibits its DNA-binding activity while dephosphorylation reverses this inhibition. Phosphorylation of cJun on sites located within its activation domain increases its ability to activate transcription without affecting its DNA binding activity. The signalling pathways that modulate the phosphorylation of these sites and their regulation will be discussed.

BZ 021 THE ACTIVATION OF THE HSP70 PROMOTER BY E1A COINCIDES WITH A RELEASE OF TBP FROM AN INTERACTION WITH THE INHIBITORY FACTOR *Dr1*, Virginia B. Kraus[1], Juan A. Inostroza[2], Danny Reinberg[2], Elizabeth Moran[3], and Joseph R. Nevins[1], [1]Section of Genetics, Howard Hughes Medical Institute, Duke University, Durham, N. C. [2]Robert Wood Johnson Medical School, UMDNJ, Piscataway, N. J.; [3]Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Recent studies have shown that the adenovirus E1A$_{12S}$ protein (the oncogenic E1A product) can *trans*-activate transcription by releasing the E2F transcription factor from inhibitory complexes with proteins such as the retinoblastoma gene product. However, E2F cannot be the only target for E1A$_{12S}$ activation since several cellular promoters have been found to be activated by the E1A$_{12S}$ protein despite the fact that they lack E2F sites. Indeed, we show that activation of the hsp70 promoter by the E1A$_{12S}$ product requires the TATAA sequence. Moreover, whereas activation of E2 transcription via E2F requires the CR1 and CR2 domains of E1A, activation of the hsp70 promoter requires the N terminal domain of the E1A protein and does not require the CR2 sequences. We conclude that the targeting of distinct transcription factors, leading to *trans*-activation of transcription of multiple promoters, involves distinct domains of the E1A proteins that are also required for oncogenic activity. Given the fact that the TATAA element appears to be the target for the activation of the hsp70 promoter, we have addressed the possibility that interactions with the TATAA-binding protein TBP might be altered by E1A. Recent experiments have identified a factor termed *Dr1* that interacts with and inhibits the transcriptional activity of TBP. We find that the E1A$_{12S}$ protein can disrupt the interaction of *Dr1* with TBP, allowing TBP to interact with TFIIA. This disruption is dependent on the N terminal E1A sequences that are also essential for trans-activation of the hsp70 promoter. It would thus appear that the activation of hsp70 through the TATAA element may be mechanistically similar to the activation of the E2 promoter via E2F, in each case involving a release of the responsible transcription factor from an inactive complex.

Phosphorylation of Transcription Factors-II (Joint)

BZ 022 IDENTIFICATION OF SIGNALS FOR PHOSPHORYLATION OF RNA POLYMERASE AND TRANSCRIPTION FACTORS, William S. Dynan, Arik Dvir, and Scott R. Peterson. Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309-0215.

We report here the purification and characterization of a template-associated protein kinase that phosphorylates the C-terminal domain (CTD) of the largest subunit of RNAP II. Prior biochemical studies have shown that the CTD is phosphorylated at about the same time that transcription begins, suggesting that phosphorylation might provide a signal that allows entry into the elongation phase of the reaction. Separately, genetic studies have shown that the CTD is involved in the response to certain DNA-binding transcriptional activator proteins. Taken together, these observations raise the intriguing possibility that some activator proteins might work by stimulating a CTD kinase, which would then transduce an activation signal by phosphorylating the RNA polymerase. The properties of the kinase that we have isolated suggest that it may participate in such a signal transduction process.

We developed a specific assay for the template-associated kinase and used this assay to purify the kinase to apparent homogeneity from human cells. The purified kinase phosphorylates the CTD of native RNA polymerase in a reaction that is dependent on DNA and the general transcription factors TFIID (TBP), TFIIB, and TFIIF. The kinase has two components. One is catalytic, and appears to be composed of a single 350 kDa polypeptide. The other is a DNA-binding regulatory component that functions to recruit the catalytic component to the DNA. This regulatory component has been identified as Ku autoantigen, based on the molecular weights of its component polypeptides, its DNA binding properties, and its reactivity with anti-Ku monoclonal antibodies. Ku autoantigen interacts with DNA by a characteristic bind-and-slide mechanism. Purified Ku autoantigen has previously been shown to function as a transcriptional activator protein, although specific DNA sequences are not required, and the mechanism by which Ku acts has therefore been uncertain. We suggest Ku protein tethers the kinase catalytic subunit to DNA, and that the ability of Ku protein to slide along DNA provides a mechanism for bringing the kinase to the transcription complex.

Separate experiments show that, under certain circumstances, the transcriptional activator protein, GAL4-VP16, can replace Ku protein as the kinase regulatory component. GAL4-VP16 stimulates the ability of the purified kinase catalytic component to phosphorylate a CTD fusion protein in vitro. Both the GAL4 and VP16 portions of the activator protein appear to be involved in the interaction with the kinase. The VP16 portion of the protein is itself phosphorylated in the reaction.

Stimulation of CTD phosphorylation is a novel and potentially general mechanism of action for transcriptional activator proteins.

BZ 023 THE REGULATION OF TRANSCRIPTION DURING THE CELL CYCLE, Nathaniel Heintz, Franca LaBella, Rosanna Martinelli and Neil Segil. Howard Hughes Medical Institute, The Rockefeller University, New York, New York.

Transcriptional induction of histone gene expression during the S phase of the mammalian cell cycle involves coordinate activation of a set of transcription factors which interact with subtype specific consensus sequences within the histone gene promoters. In the case of histone H2b genes, the transcription factor Oct1 binds to the S phase regulatory sequences and mediates cell cycle regulation. Recent investigations have established that Oct1 undergoes a complex program of phosphorylation during the cell cycle which correlates with changes in its functional activity. Both in vivo and in vitro studies have established that mitotic hyperphosphorylation of Oct1 at ser^{385} within the homeodomain results in inhibition of DNA binding, and that this residue is specifically phosphorylated by PKA in vitro. These results demonstrate that Oct1 activity is modulated by phosphorylation during the cell cycle, and suggest that characterization of additional posttranslational modifications to Oct1 that occur during the cell cycle may shed light on transcriptional regulation by the POU domain proteins.

To assess whether other transcription factors which modulate histone gene transcription during the cell cycle are regulated by mechanisms similar to those controlling Oct1 activity, we have extended these studies to a second histone gene regulatory factor. H1TF2 is a novel, heterodimeric CCAAT binding protein which participates in histone H1 cell cycle control. Analysis of H1TF2 phosphorylation in vivo has established that it is also modified late in the cell cycle, and that its phosphorylation during mitosis also results in loss of DNA binding activity. Thus, two functionally related but entirely distinct transcription factors involved in temporal regulation of transcription during the cell cycle appear to be coordinately regulated by common molecular mechanisms. These results provide a biochemical framework for further work on the coordinate regulation of transcription during the mammalian cell cycle, and suggest that phosphorylation may be a key regulatory step in modulating the activities of these factors and their close relatives under other biological circumstances.

BZ 024 IRF-1 AND IRF-2 ; A LINK BETWEEN THE INTERFERON SYSTEM AND CELL GROWTH CONTROL. Tadatsugu Taniguchi, Hisashi Harada, Nobuyuki Tanaka, Motoo Kitagawa, Takashi Fujita*, Nobumasa Watanabe, Jun Sakakibara, Hitomi Yamamoto, Takatoshi Kawakami.

Cytokines regulate cell growth in a positive or negative manner by inducing their respective target genes. Interferons(IFNs) represent a family of cytokines with many biological activities including anti-proliferative activity. In the process of analyzing the mechanism of the IFN-β gene expression, we have identified specific DNA motifs in the promoter region of the gene. The motifs include the ubiquitous NFκB site, and Oct-1 site, and the other is more specific to the IFN genes characterized particularly by the presence of the hexamer AAGTGA motif. We have found specific nuclear factors interacting with this motif, IRF-1 and IRF-2 molecules. The functional role of IRF-1 and IRF-2 is getting clearer. There is ample evidence on the involvement of these nuclear factors in the IFN-β gene induction. In fact, evidence has been provided that, following viral induction, IRF-1 is synthesized and phosphorylated to act on the IFN-β promoter. We detect in the nuclear extract of NDV-infected mouse L929 cells a factor complex which involves IRF-1 and shown a higher affinity to the IFN-β promoter than IRF-1 or IRF-2(monomer). Our experimental data suggest that the complex is heterodimer which consists of IRF-1 and IRF-2, and it interacts with the promoter involving a larger DNA sequence element than either IRF-1 or IRF-2.

In the context of the regulation of cell growth by IFNs, it is interesting that the IRF-1 gene is IFN-inducible *per se*. Hence it may be one of the target genes for the anti-proliferative function of IFNs. Recently, we have proveded evedence that restained cell growth depend upon the balance between these two competitive factors. In fact, the IRF-1/IRF-2 expression ratio oscillates during the cell cycle, and perturbation of this ratio results in profoundly altered growth properties of NIH3T3 cells. These findings suggest a novel mechanism in the regulation of cell growth and imply the involvement of the two mutually-antagonistic transcription factors in oncogenesis.

* *Present address : The Rockefeller University, USA*

Phosphatases

BZ 025 STRUCTURE AND FUNCTION OF NON-TRANSMEMBRANE TYROSINE PHOSPHATASES (PTPs), Benjamin G. Neel, John V. Frangioni, Victor Shifrin, Ulrike Lorenz, Jorge Plutzky, Robert M. Freeman Jr., Pamela Beahm, Sharon Chang, and Robert J. Lechleider, Molecular Medicine Unit Unit, Beth Israel Hospital, Boston, MA. 02215

We have molecularly cloned and studied the involvement of three non-transmembrane PTPs in cellular signal transduction. PTP1B is targeted to the ER via a 35 amino acid C-terminal domain and is further modified in cycling and non-cycling cells. In human fibroblasts stimulated from quiescence by serum or a number of peptide growth factors, a novel alternative PTP1B transcript is produced. RNase protection experiments establish that this transcript results from *suppressed splicing* of the last intron, leading to an altered C-terminus. The biologic consequences of this alternative splice product are under investigation. Moreover, during the G2->M transition, PTP1B undergoes hyperphosphorylation on serine, resulting in a protein of decreased mobility and altered in vitro activity. In human platelets, which are post-mitotic, PTP1B is also subject to two types of modification. First, it is rapidly phosphorylated (onset 15 sec) on a different serine residue upon thrombin stimulation. The same residue appears to be phosphorylated in response to TPA. Second, treatment with calcium ionophores results in calpain-depedent cleavage of the C-terminal 5 kD of PTP1B and activation of its PTP activity. Taken together, these results suggest that the PTP1B C-terminus plays a regulatory role in addition to its targetimg function.

We have also studied two SH2-containing PTPs. SHPTP1 is expressed predominantly in hematopoietic cells. Its expression is strongly induced early during hematopoietic differentiation of ES cells in vitro. Using affinity-purified anti-SHPTP antibodies, we have found that it is a predominantly cytosolic 65 kD phosphoprotein. In the IL-3 dependent BaF3 cell line, it is phosphorylated on serine. There appears to be a slight increase in serine phosphorylation upon IL3 stimulation. Western blotting of SHPTP1 immunoprecipitates reveals coprecipitation of several larger phosphotrysoyl proteins, which vary at different times following stimulation. In contrast, in cells expressing high levels and/or activated versions of src family kinases, SHPTP1 appears to be serine and tyrosine phosphorylated. Moreover, in LSTRA cells, SHPTP1 coprecipitates with a single, as yet unidentified 160 kD tyrosyl phosphoprotein. Conversely, SHPTP2 is ubiquitously expressed. Sequence analysis strongly suggests that it is the mammalian homolog of Drosophila csw, which functions in concert with D-raf to positively transduce the signal of the torso receptor tyrosine kinase. We have raised antibodies to SHPTP2 and identified it as a 68 kD protein. Experiments exploring association between SHPTP2 and several known cellular phosphotyrosyl proteins will be presented.

Signal Transduction and Disease (Joint)

BZ 026 FUNCTION OF THE RETINOBLASTOMA PROTEIN Wen-Hwa Lee Center for Molecular Medicine/ Institute of Biotechnology, University of Texas Health Science Center, San Antonio, Texas , 78245

A class of cellular genes in which loss-of-function mutations are tumorigenic has been proposed. The RB gene (RB) appears to operate in exactly this fashion. Consistent with its ubiquitous expression pattern, RB gene inactivation is not only limited to retinoblastomas. Many other cancers such as osteosarcoma, breast carcinoma, small cell lung carcinoma, bladder carcinoma and prostate carcinoma also contain inactivated RB genes. We have introduced, via retroviral-mediated gene transfer, a cloned RB gene into retinoblastoma, osteosarcoma, prostate carcinoma, bladder carcinoima and breast carcinoma cells that have inactivated endogenous RB genes. Expression of the exogenous RB gene consistently suppressed their tumorigenecity in nude mice. These results indicate that the RB gene is a general suppressor gene of multiple type of cancer cells.

To understand the biological function of RB, we have established mice model to address the role of RB during the developmental process. Our results suggest that mice without normal RB protein expression are embryonic lethal probably due to the ectopic mitosis following cell death in neuronal and hematopoetic systems. With only one copy of RB gene, mice appear to be phenotypically normal with brian tumor at the later age of about 10 months. However, overexpression of RB protein through its own promoter resulted in dwarfism of mice. The degree of dwarfism was inversely proportional to the amount of transgenic RB protein expressed. Delay of developmental stage was observed in mice with highest transgenic RB protein expressed (about 2 folds to its endogenous RB protein). These results indicated that the RB gene clearly plays an important role during the developmental process.

To further understand the biochemical function of RB, RB protein was expressed in E. coli or in insect cells and purified to a homogeneity. Microinjection of this protein into cells at different stages inhibits G1 progression of cell cycle, suggesting that the protein may be functional at the stage of G0 to early G1. The pure RB protein has an intrinsic property of forming multimer which was regulated by phosphorylation. This study provided a novel concept how RB regulating other proteins in a coordinate manner. Indeed, many endogenous cellular proteins exist that bind to the RB and thereby may mediate its function. Characterization of this group of proteins should shed light on what is the role of RB in the cell

Tyrosine Protein Kinase Receptors and Their Substrates

BZ 100 CHARACTERIZATION OF NOVEL TKs INCLUDING MEMBERS OF THE FGF and VPF/VEGF RECEPTOR FAMILIES

Kari Alitalo, Juha Partanen, Mathias Bergman, Olga Aprelikova, Satu Vainikka, Elina Armstrong, Kay Huebner*, Jaana Korhonen, Riitta Alitalo and Katri Pajusola Pathology and Virology and Transplantation Laboratory, Univ. Helsinki, 00290 Helsinki, Finland and *Jefferson Cancer Institute, Philadelphia, PA; USA

In order to get insight into the growth regulation of hematopoietic and leukemia cells and to characterize key signalling molecules in their differentiation, we have cloned novel tyrosine kinase (TK) cDNAs from human leukemia cells having a bipotential erythroid/megakaryoblastoid differentiation potential. This resulted in the identification of two novel TKs of the fibroblast growth factor receptor family (FGFR-3 and FGFR-4) and one TK of vascular endothelial growth factor (VEGF/VPF, SCF/CSF-1/PDGF) receptor family. One of the novel TKs encodes a receptor expressed in endothelial cells of mouse embryos and induced in adult mice during neovascularization associated with superovulation and wound healing. In addition, we have characterized a cytoplasmic TK, CSK, which is abundant in platelets and capable of downregulating the activity of TKs of at least the *src* family. We are studying the possibility that CSK is a product of an anti-oncogene.

Proc. Natl. Acad. Sci. 87: 8913, 1990; *EMBO J.* 10: 1347, 1991; 11: 2919, 1992; in press; *Oncogene* 6: 2013, 1991; *Nucl. Acids Res.* 19: 5096,1991; *Mol. Cell Biol.* 12: 1698, 1992; *Cancer Res.* 52: 2004; 52: 2004, 1992; *Progr. Growth Factor Res.* 4: 69, 1992.; *Cytogen. Cell Gen.*, in press,

BZ 101 Characterization of the *FLT4* receptor tyrosine kinase

Olga Aprelikova, Katri Pajusola, Jaana Korhonen, Arja Kaipainen, Liisa Pertovaara, Riitta Alitalo and Kari Alitalo Cancer Biology Laboratory, Departments of Virology and Pathology, and Transplantation Laboratory, University of Helsinki, 00290 Helsinki 29, FINLAND

The *FLT4* receptor tyrosine kinase cDNA was identified from human HEL erythroleukemia cell library by polymerase chain reaction-amplification. We previously reported a partial sequence of *FLT4* and showed that the *FLT4* gene maps to chromosomal region 5q33-qter (1). Here we present the full-length sequence of the predicted *FLT4* protein. The extracellular domain of *FLT4* consists of seven immunoglobulin-like loops including twelve potential glycosylation sites. On basis of structural similarities *FLT4* and the previously known *FLT1* and *KDR/FLK1* receptors constitute a subfamily of class III tyrosine kinases. *FLT4* was expressed as 5.8 kb and 4.5 kb mRNAs which were found to differ in their 3' sequences and to be differentially expressed in the HEL and DAMI leukemia cells. *Interestingly, a Wilm's tumor cell line, a retinoblastoma cell line and a* nondifferentiated teratocarcinoma cell line expressed *FLT4*, whereas differentiated teratocarcinoma cells were negative. Most fetal tissues also expressed the *FLT4* mRNA, with spleen, brain intermediate zone and lung showing the highest levels. In *in situ* hybridization and immunohistochemistry the *FLT4* was located in bronchial epithelial cells of fetal lung. No evidence was obtained for expression of *FLT4* in endothelial cells of blood vessels.

BZ 102 TRANSDUCTION OF CIRCULAR MEMBRANE RUFFLING BY THE PDGF β-RECEPTOR IS DEPENDENT ON ITS KINASE INSERT,

Ann-Kristin Arvidsson, Carl-Henrik Heldin and Lena Claesson-Welsh, Ludwig Institute for Cancer Research, Box 595, S-751 24 Uppsala, Sweden.

The platelet-derived growth factor (PDGF) α- and β-receptors both mediate a mitogenic response, but only the β-receptor mediates circular actin reorganization and chemotaxis. The tyrosine kinase domains of the receptors contain non-catalytic inserts of about 100 residues. In order to determine the role of these domains in the differential signalling of the two receptors we constructed chimeric PDGF receptors and expressed the constructs in porcine aortic endothelial (PAE) cells. The chimeric PDGF α-receptor, containing the kinase insert from the β-receptor instead of the endogenous α-receptor kinase insert, was able to mediate actin reorganization in the form of circular membrane ruffling. The corresponding chimeric PDGF β-receptor, containing the α-receptor kinase insert, failed to mediate this response. However, both types of chimeric receptors transduced a mitogenic response. These data indicate that the PDGF β-receptor kinase insert is a prerequisite for induction of circular membrane ruffling. Analysis of receptor-associated substrates by in vitro kinase assay revealed that certain phosphoproteins associated specifically with the PDGF β-receptor kinase insert. Our current work is aimed at determining the binding site(s) for these substrates within the kinase insert domain and further analysis of their role in signal transduction leading to circular membrane ruffling.

BZ 103 MOLECULAR CHARACTERIZATION OF THE MOUSE HOMOLOGUE OF THE *C. elegans sem-5* GENE.

Mariano Barbacid, Ki-Ling Suen, and Xosé R. Bustelo, Department of Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ, 08543

In an effort to identify murine genes related to the SH2/SH3-containing *vav* proto-oncogene, we conducted a series of PCR-aided amplifications using primers specific for the various domains of the *vav* gene product. Among those cDNA sequences amplified using SH2 and SH3 primers we identified clones corresponding to a gene encoding a small protein of 217 amino acid residues with an overall SH3-SH2-SH3 structure highly reminiscent of the carboxy terminus of the *vav* gene product. Sequence comparison of the predicted protein revealed a high degree of homology to the human Grb-2 protein (99% identity) and the product of the *C. elegans sem*-5 gene (59% identity, 74% homology), thus suggesting that this novel gene is the murine homologue of the *sem*-5/*grb*-2 locus. The human *grb*-2 gene was recently identified by screening expression libraries with a tyrosine-phosphorylated carboxy-terminal tail of the EGF receptor (Cell, **70**, 431, 1992). The *C. elegans sem*-5 gene mediates signal transduction between the Let-23 tyrosine kinase receptor and the downstream Ras-like Let-60 protein, a process essential for sex myoblast migration, differentiation of cells within the vulval lineage, and larval survival (Nature, **356**, 340, 1992). Northern blot and *in situ* hybridization analysis indicate that the murine *sem*-5/*grb*-2 gene is widely expressed during embryonic development and in adult tissues. Biochemical studies have shown that the Sem-5/Grb-2 protein can interact with a number of tyrosine-phosphorylated molecules, including ligand-activated tyrosine kinase receptors (EGF and PDGF receptors), cytoplasmic tyrosine kinases of the *src* gene family, and other SH2-containing proteins such as the product of the *shc* gene. These interactions appear to be mediated exclusively by its SH2 sequences. These results identify the Sem-5/Grb-2 protein as a common mediator of signal transduction in a wide variety of cell types.

BZ 104 IDENTIFICATION OF TYROSINE PHOSPHORYLATION SITES IN THE KINASE INSERT DOMAIN OF THE PDGFR α SUBUNIT, Chantal E. Bazenet and Andrius Kazlauskas, National Jewish Center, Department of Pediatrics, 1400 Jackson Street, Denver, CO 80206.

Previous studies have shown that activation of the intrinsic kinase activity of the PDGFR β subunit and subsequent tyrosine phosphorylation are central to signal transduction mediated by the PDGFR β subunit. Like the β subunit, the α subunit encodes a tyrosine kinase that is activated and tyrosine phosphorylated upon binding of PDGF, so it seems probable that tyrosine phosphorylation of the α subunit plays a role in intracellular signal relay as well. To address this possibility, we have started to identify the sites of tyrosine phosphorylation of the α subunit. So far, we have found three tyrosine residues phosphorylated *in vitro* as well as *in vivo*. These tyrosine residues are located in the kinase insert domain of the receptor at the positions 720, 731, and 742. Y731 and Y742 are homologuous toY740 and to Y751, respectively, in the β PDGFR subunit that are responsible for binding of phosphatidylinositol-3 kinase (PI3K). Mutation at Y742 significantly decreases the amount of PI3K that associates with kinase insert-containing fusion proteins, whereas mutation at Y731 reduces PI3K binding to an undetectable level, reproducing the results of Yu *et al*, (1) using the intact receptor. Y720 does not have any homologuous tyrosine in the β PDGFR subunit and therefore could have a very specific function in the PDGFR α subunit. Substitutions of tyrosine 720, 731, and 742 with phenylalanine have been made and the mutants are beeing expressed in TRMP cells.

(1) Yu *et al*, Mol. Cell. Biol. (1991) 11 3780-3785.

BZ 105 THE CLONING OF 5 cDNAs ENCODING PUTATIVE EMBRYONIC TYROSINE KINASES, Leslie G. Biesecker, Lisa R. Gottschalk, and Stephen G. Emerson. Departments of Pediatrics and Internal Medicine, University of Michigan School, Ann Arbor, MI, 48109.

A growing list of growth factors and cytokines have been implicated in the differentiation of the mammalian embryo. Several of these ligands mediate their function through receptors that belong to the protein kinase family. To characterize the molecular regulation of early mammalian development we have isolated additional members of this class of kinases from embryonic tissue. We have made use of the differentiation potential of the murine embryonic stem cell system to clone 5 partial cDNAs that encode putative embryonic tyrosine kinases (ETKs). Embryonic stem cells (ESD3) were differentiated in the presence of human umbilical cord serum to induce hematopoiesis. RTPCR was performed using degenerate primers for highly conserved tyrosine kinase domains VI & IX. The PCR products were cloned into pUC18, sequenced and compared to the Genbank/EMBL database. Of 96 clones, 78 were of the predicted size and 52 were identical with previously cloned tyrosine kinases. Sixteen of the clones conformed to tyrosine kinase motifs and represented 5 apparently novel PTKs, designated as ETK-1 through 5 (Embryonic tyrosine kinase). One of these murine ETKs (ETK-4) is very similar to the recently reported human gene KDR (kinase domain receptor). Using Northern blot analysis, an ETK-1 cDNA probe hybridized to transcripts of 3.5 and 3.0 kb and both were present in heart, brain, liver, lung, spleen, muscle, kidney, and testicle (highest in the kidney). The single ETK-2 transcript was 4.6 kb and was highly expressed in brain, was moderately expressed in the testicle, and weakly expressed in the lung. The ETK-3 cDNA probe hybridized to transcripts of 2.7 and 3.2 kb and, like ETK-1, was expressed in all tissues studied. Interestingly, ETK-3 lacks the Asp-Phe-Gly motif that has been thought to be invariant in the protein kinase family. ETK-4 is also expressed only at low levels in each of these tissues. ETK-5 is expressed at moderate levels in heart, brain, lung, and kidney and at low levels in spleen, liver, and testis. Because of their more restricted pattern of expression, ETK-2 and ETK-5 were chosen for further study. The full length cDNA sequences of ETK-2 and ETK-5 will be presented. Future investigations will focus on the function of these putative ETKs.

BZ 106 DIFFERENT SIGNALING PATHWAYS FROM THE HUMAN C-*KIT*/SCF RECEPTOR MEDIATE PROLIFERATION AND MOTILITY

Peter Blume-Jensen, Lars Rönnstrand, Agnetha Siegbahn and Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Biomedical Center, BOX 595, S-751 24 UPPSALA, Sweden.

Transmembrane tyrosine kinase receptors are typically activated by ligand-induced oligomerization, which leads to receptor autophosphorylation, as well as phosphorylation of other cellular proteins. Some of the proteins phosphorylated by tyrosine kinase receptors, e. g. ras GTPase activating protein (GAP), phosphatidylinositol (PI) 3'kinase, $p60^{c\text{-}src}$ and phospholipase C-γ (PLC-γ), associate with the autophosphorylated receptors through their src-homology 2 (SH2) domains. These in turn activate other substrates downstream, whereby a signaling cascade is initiated leading to the ultimate responses of proliferation, differentiation or motility. Recently, it has been shown, that many of these pathways converge on the MAP-2 kinase family of serine/threonine kinases.

We have examined the signaling properties of the human c-*kit*/SCF receptor, which is structurally related to the platelet-derived growth factor (PDGF) receptor subfamily of tyrosine kinases, and explored its possible interaction with the protein kinase C (PKC) signaling pathway. PKC mediated receptor phosphorylation on S/T residues , which was inhibitory for autophosphorylation on tyrosine residues, and association with certain substrates. MAP-2 kinase activation by the c-*kit*/SCF receptor in relation to the PKC pathway was also investigated. The consequenses of this interaction with regard to cell proliferation and cell motility has been investigated.

BZ 107 EGF-INDUCED SIGNAL TRANSDUCTION: INVOLVEMENT OF THE CYTOSKELETON, J. Boonstra, J.C. den Hartigh, B. Payrastre, P.J. Rijken, A.J. Verkleij and P.M.P. van Bergen en Henegouwen, Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.

Recently we have demonstrated that the high-affinity class of EGF-receptor is associated to the cytoskeleton. Furthermore, evidence is presented that the high-affinity EGF-receptor class is responsible for EGF-induced signal transduction. These findings suggest a role for the cytoskeleton in EGF-induced signal transduction.

Using selective extraction protocols, we demonstrated that the EGF-receptor is associated primarely to the actin microfilament system. Using purified EGF-receptors and purified actin, it is demonstrated that the EGF-receptor is associated directly to actin with no other actin-binding proteins involved. In addition, the actin-binding domain of the EGF receptor is identified by competition studies with specific antibodies and synthetic peptides.

In addition to the EGF receptor, a number of other components involved in signal transduction are associated to the cytoskeleton. It is shown that PI-kinase, PIP kinase, diacylglycerol kinase and phospholipase C activities are associated to the actin microfilament system.

Activation of the EGF-receptor by binding of EGF causes a strong reorganization of the actin microfilament system. Using confocal scan laser microscopy, it is shown that stress fibers disappear and F-actin fibers appear in the cortical area of the cells.

After activation of the EGF-receptor at the membrane level, the signals are transduced to the nucleus. In this respect it is of interest that components of the PI cycle are present in the nucleus as well. After selective extraction and purification of the nuclear matrix, we have shown that PI kinase is present in the peripheral matrix while PIP kinase, PLC and diacylglycerol kinase are associated to the internal matrix.

The data presented suggest that the cytoskeleton plays an important role in EGF-induced signal transduction, either by actin as a matrix in the formation of signal transduction complexes at the plasma membrane or/and as a matrix for the transduction of the signal from the membrane to the nucleus.

BZ 108RAS-ACTIVATION BY INSULIN AND EGF THROUGH ENHANCED EXCHANGE OF GUANINE NUCLEOTIDES ON p21RAS. Johannes L. Bos, René H. Medema, Boudewijn M.T. Burgering, Gijsbertus J. Pronk, Alida M.M. de Vries-Smits, Pascale C. van Weeren, Jan-Paul Medema and Loesje van der Voorn. Utrecht University, Laboratory for Physiological Chemistry, Vondellaan 24a, 3521 GG Utrecht, The Netherlands.

A number of growth factors, including insulin and epidermal growth factor (EGF), induce an accumulation of the GTP-bound form of p21ras. This accumulation could either be caused by an increase in guanine nucleotide exchange on p21ras or by a decrease in the GTPase activity of p21ras. To investigate whether insulin and EGF affect nucleotide exchange on p21ras we measured binding of [a^{32}P]-GTP to p21ras in cells permeabilized with streptolysin O. For this purpose we used a cell line expressing elevated levels of p21Hras and highly responsive to insulin and EGF. Stimulation with insulin or EGF resulted in an increase in the rate of nucleotide binding to p21ras. To determine whether this increased binding rate is due to activation of a guanine nucleotide exchange factor, we made use of the inhibitory properties of a dominant negative mutant of p21ras, p21ras(Asn17). Activation of p21ras by insulin and EGF in intact cells was abolished in cells infected with a recombinant vaccinia virus expressing p21ras(Asn17). In addition, the enhanced nucleotide binding to p21ras in response to insulin and EGF in permeabilized cells was blocked upon expression of p21ras(Asn17). From these data we conclude that insulin and EGF activate p21ras through an increase in the activity of a guanine nucleotide exchange factor.

BZ 109 THE EFFECT OF TYROSINE KINASE INHIBITORS ON EGF-DEPENDENT PROLIFERATIVE AND PHOSPHORYLATION EVENTS IN HUMAN SQUAMOUS CARCINOMA CELL LINES, Valerie G. Brunton*, David Robins+ and Paul Workman*, Department of Medical Oncology* and Chemistry+, University of Glasgow, Switchback Road, Bearsden, Glasgow G61 1BD, UK.

There is increasing evidence that the EGF receptor (EGFR) plays an important role in the development and regulation of human squamous cell carcinomas. The EGFR is overexpressed in many human squamous cell carcinoma cell lines and tumours. This is due to amplification of the EGFR gene. We have been studying the effect of tyrosine kinase inhibitors on the growth and EGF-dependent phosphorylation events in two human squamous cell carcinoma cell lines: A431 and B2A4 (HN5 clone). Both cell lines have high levels of the EGFR (3 x 10^6 and 5 x 10^6 per cell respectively). A431 proliferation is inhibited by EGF (10ng/ml) whereas the proliferation of B2A4 cells is stimulated by the same concentration of EGF. Two tyrosine kinase inhibitors termed tyrphostins were used in the study: RG 50864 and RG 13022. Both these compounds were able to inhibit the EGF-dependent proliferation of B2A4 cells but were unable to reverse the growth inhibitory effect of EGF on A431 cells. This suggests that the tyrphostins may preferentially inhibit the tyrosine phosphorylation of substrates for the EGFR tyrosine kinase which are involved in the proliferative signal but not those involved in triggering inhibition in A431 cells. To study this we have used antiphosphotyrosine antibodies to determine the effect of the two inhibitors on autophosphorylation of the EGFR and also phosphorylation of other cellular substrates. RG 13022 was a more effective inhibitor of EGFR autophosphorylation than RG 50864 whereas in a cell-free EGFR tyrosine kinase assay there was no such distinction. The IC_{50} of RG 50864 in this latter assay was significantly lower than for inhibition of EGFR autophosphorylation. The time course of inhibition by the two inhibitors was also characterised. Currently we are examining the effects of the inhibitors on the phosphorylation of different proteins following activation of the EGFR in the two different cell lines.

BZ 110 RECEPTOR BINDING PROPERTIES AND MITOGENIC EFFECTS OF INSULIN, INSULIN ANALOGUES AND HUMAN GROWTH HORMONE ON A MURINE LYMPHOID T-CELL LEUKEMIA LINE DEVOID OF IGF I RECEPTORS.

Claus T Christoffersen§, Dvorah Ish-shalom*, Mapoko Ilondo§,Nina Sacerdoti-Sierra*, David Naor*, Pierre De Meyts§. The Hagedorn Research Institute, DK 2820 Gentofte, Denmark§ and The Lautenberg Center for General and Tumor Immunology, The Hebrew University-Hadassah Medical School, Jerusalem 91910, Israel*.

Previous work has shown that the *in vitro* proliferation of the spontaneous lymphoid T-cell leukemia designated LB was enhanced by a wide range of insulin concentrations, including the low physiological, but not by IGF I or IGF II (Pillemer, G., Lugasi-Evgi, H., Scharovsky, G. and Naor, D., Int. J. Cancer, 50: 80-85,1992). We have now investigated in more detail the characteristics of cell surface receptors on the LB cells. No specific IGF I binding was detected. The cells contained 3000 insulin receptors with a typical pH and temperature dependence of binding, dissociation of labelled insulin accelerated by unlabelled insulin, and a typical insulin dose-response curve for negative cooperativity. Insulin analogues showed relative binding potencies similar to those seen on classical insulin target cells. Analogues with a slower dissociation rate than insulin showed a disproportionately enhanced mitogenic potency as determined in dose-response curves for thymidine incorporation.

The LB cells showed also a marked enhancement of proliferation with human growth hormone. The cells express 650 binding sites for growth hormone with a typical affinity and nearly full competition with bovine growth hormone , suggesting somatogenic rather than lactogenic receptors. The absence of IGF I receptors make this cell line an ideal model for investigating the signalling pathways for the mitogenic effects of insulin and growth hormone mediated through their own receptors.

BZ 111 **Characterization of a distinct subpopulation of PDGF receptors.** Steven R. Coats, W.J. Pledger and T.O. Daniel. Dept. of Cell Biology, Vanderbilt University School of Medicine, Nashville, TN 37212.

The pool of PDGF receptors present in Balb/c-3T3 fibroblasts has previously been thought to consist of integral membrane proteins which are soluble in non-ionic detergents. We have solubilized the triton-insoluble pellet in SDS or urea and determined that up to 40% of the total PDGF β receptor population remained in a triton-insoluble form. The triton-insoluble β receptors maintain a low basal state of tyrosine phosphorylation. However, in the presence of ligand there is an increase in the level of phosphorylated tyrosine residues in both the triton-soluble (S) and triton-insoluble (I) β receptor. The addition of PDGF down regulates the triton-soluble receptors while stabilizing the triton-insoluble receptors. The triton-insoluble receptors are not accessible to either trypsin or neuraminidase when these agents are placed directly on the cells at 4°C, indicating that the triton-insoluble receptors are not localized to the cell surface. Additional experiments revealed that movement of receptors from the triton-soluble to the triton-insoluble receptor pool occurs. Collectively, these data demonstrate that a pool of previously uncharacterized PDGF receptors, possessing a distinct kinetic pattern, exists within Balb/c-3T3 fibroblasts.

BZ 116 PLATELET-DERIVED GROWTH FACTOR HOMO AND HETERO DIMERS STIMULATE PHOSPHATIDYLINOSITOL 3 KINASE IN HUMAN GLOMERULAR MESANGIAL CELLS,
Goutam Ghosh Choudhury, Purba Biswas and Hanna E. Abboud, Department of Medicine, University of Texas Health Science Center at San Antonio, San Antonio, TX 78284

Glomerular inflammation and local production of growth factors in the microenvironment of the mesangium leads to mesangial cell proliferation. One potent mesangial cell mitogen is platelet-derived growth factor (PDGF). The precise mechanism of PDGF-induced mesangial cell mitogenesis is not clear. Activation of a novel signal transducing enzyme, phosphatidylinositol 3 kinase (PI 3 kinase) has been associated with transforming protein tyrosine kinases and activated receptor tyrosine kinases. We studied the activation of PI 3 kinase in response to all three isoforms of PDGF BB, AA homodimer and AB heterodimer in antiphosphotyrosine immunoprecipitates. PDGF BB induced 10 to 45 fold stimulation of PI 3 kinase activity. HPLC analysis of the deacylated PI 3 kinase reaction product proved the authenticity of PI 3 phosphate. Time course study with PDGF BB revealed biphasic kinetics with an increase in PI 3 kinase activity at 2 minutes followed by a second peak at 15 minutes. At 30 minutes 67% of total peak PI 3 kinase activity is lost. PDGF AA also stimulated PI 3 kinase activity at 5 minutes and at 30 minutes the activity was decreased to almost basal. Comparison of PI 3 kinase activation with different PDGF isoforms showed a decreasing order of potency: PDGF BB > AB > AA. Mitogenic potential of each isotype also revealed the same order of potency. Immunoprecipitation of PDGF α and β receptors with specific monoclonal antibodies from PDGF-stimulated mesangial cells showed the presence of PI 3 kinase activity in the immunbeads indicating the association of this enzyme with the receptor. Immunoprecipitation and subsequent immunokinase assay of PI 3 kinase from PDGF-treated mesangial cells also confirmed the association of PDGFR with this novel enzyme.

These data suggest that in mesangial cell all three isoforms of PDGF stimulate the novel PI 3 kinase to produce D3 phosphorylated inositides and that this enzyme may play a crucial role in PDGF-induced mesangial cell proliferation during glomerular injury.

BZ 117 TRANSFER OF THE MOTOGENIC AND INVASIVE RESPONSE TO SCATTER FACTOR/HEPATOCYTE GROWTH FACTOR BY TRANSFECTION OF THE HUMAN *c-MET* PROTO-ONCOGENE
Silvia Giordano, Zhu Zhen, Enzo Medico, Giovanni Gaudino, Francesco Galimi and Paolo M. Comoglio
Department of Biomedical Sciences and Oncology, University of Torino Medical School, 10126 Torino, Italy

The *c-MET* proto-oncogene encodes p190^{MET}, a tyrosine kinase which is the receptor for a molecule known as Scatter Factor or Hepatocyte Growth Factor (SF/HGF). This molecule has different biological activities, including stimulation of cell motility, promotion of matrix invasion and, in some cells, mitogenesis. We have cloned the full length *c-MET* cDNA and transfected it into NIH 3T3 fibroblasts. Stable transfectants expressed the p190^{MET} receptor, together with two previously described truncated forms of 140 and 130 kDa, lacking the tyrosine kinase domain. All three forms bound radiolabelled SF/HGF. SF/HGF stimulated tyrosine kinase activity of the transfected p190^{MET}, and induced changes in cell shape, migration in Boyden chambers and invasion of collagen matrices *in vitro*. The motile and invasive phenotype was transient and strictly dependent upon the presence of SF/HGF. The factor did not stimulate either cell growth or thymidine incorporation in transfected cells, while it promoted colony formation in soft agar in the presence of 5% foetal calf serum. These data show that, in the presence of its ligand, the *c-Met* receptor expressed in fibroblasts induces cells to pursue a motogenic-invasive rather than a proliferative program.

BZ 118 HEPATOCYTE GROWTH FACTOR STIMULATES RAS-GUANINE NUCLEOTIDE EXCHANGE ACTIVITY.
A. Graziani[1], D. Gramaglia[1], P. dalla Zonca[1], R. Medema[2], J. Bos[2] and P.M. Comoglio[1]. [1]Department of Biomedical Sciences & Oncology, University of Torino, Italy. [2]Laboratory for Physiological Chemistry, University of Utrecht, The Netherlands.

Hepatocyte Growth Factor (HGF), induces mitogenesis and cell scattering in epithelial and endothelial cells. Its receptor has been identified as the product of the *MET* proto-oncogene, a 190-kDa heterodimeric protein-tyrosine kinase (p190^{MET}). Ras proteins are involved in coupling membrane signalling proteins to intracellular targets. Previous genetic and biochemical evidence indicate that activation of growth factor receptor's tyrosine kinase leads Ras proteins to shift from the unactive GDP-bound state to the active GTP-bound state, by putatively stimulating a Ras-Guanine Nucleotide Exchange factor. HGF stimulates cell scattering in A549 cells, an epithelial cell line. Within 2 minutes stimulation of [^{32}P]ortophosphate labelled A549 cell, HGF increases total labelled Ras-bound GTP and GDP up to 5 to 10 times the controls. The ratio of GTP to GDP Ras-bound in the HGF stimulated cells is about 50%. Conversely treatment of [^{32}P]-ortophosphate labelled GTL 16 cells, featuring p190^{MET} constitutively active, with micromolar concentrations of a protein-tyrosine kinase inhibitor, TMO (3-(1',4'dihydroxytetralyl)methylen-2-3oxindole), lowers 5 to 10 folds the total Ras-bound Guanine Nucleotides. The Ras-Guanine Nucleotide Exchanger activity was then assayed *in situ*, in A549 cells permeabilized with digitonin in presence of [α^{32}P]-GTP. HGF treatment leads to a 5 fold increase of total labelled Ras-bound GDP and GTP. Excess of cold GTP (0.5mM) displaces the binding of both labelled GDP and GTP to Ras protein. These results show that HGF binding to its protein-tyrosine kinase receptor stimulates a Ras-Guanine Nucleotide Exchanger activity. Moreover Ras-bound GTP is rapidly hydrolyzed to GDP by a Ras-GTPase which is present in HGF stimulated cells. Thus HGF appears to promote an increased turnover of Ras protein between the GDP- and GTP- bound state, rather than merely stabilize the GTP-bound form.

BZ 119 MUTATIONAL ANALYSIS OF THE ROLE OF SER85 AND ARG86 OF THE INSULIN RECEPTOR IN INSULIN BINDING AND TYROSINE KINASE ACTIVITY.
Karen Grønskov*, Henrik Vissing*, Ronald M. Shymko*, Hans Tornqvist§, and Pierre De Meyts*, *Hagedorn Research Institute, Gentofte, Denmark and §Dept. of Medical and Physiological Chemistry 4, University of Lund, Sweden.

Insulin receptor autophosphorylation and kinase activity have been reported to be constitutively increased in fibroblasts from a patient with heritable insulin resistance (Longo, N et al, BBRC, 167, 1229, 1990), due to a homozygous mutation of Arg to Pro at position 86 in the alpha subunit of the receptor (Longo, N et al, Clin. Res., 40, 2, 239A, 1992). Our own studies have suggested that the region surrounding Phe 89 (F89) is involved in insulin binding. Computer modelling of the sequence around F89 (based on an assumed analogy with the insulin dimer) suggested that R86 may be at a beta-turn and that this position may thus be conformationally sensitive to mutations. Therefore, the following mutations were created in the human insulin receptor cDNA by site- directed mutagenesis: R86P-HIR, R86N-HIR, S85T,R86N-HIR and S85W,R86K-HIR. The receptors were stably transfected into BHK-cells and transiently expressed in 293 cells. All mutations were well tolerated except R86P-HIR. Cells expressing the R86P-HIR showed no specific insulin binding above background. Western blot analyses of WGA-purified receptors indicated that the R86P-HIR exists as a 200 kDa unprocessed receptor plus additional smaller fragments. Immunocytochemistry showed that the receptor is not expressed at the surface. Unlike the wild type receptors, which were found to incorporate ^{32}P into tyrosine residues of a 95 kDa peptide which increased upon insulin stimulation, *in vitro* phosphorylation of WGA-purified R86P-HIR receptors resulted in a 200 kDa tyrosine phosphorylated protein, of which the phosphorylation appeared to be insulin insensitive. The kinase activity on a synthetic substrate was also constitutively activated .

BZ 120 LOSS OF p185 PHOSPHOTYROSINE LEADS TO SUPPRESSION OF TRANSFORMATION IN TWO REVERTANTS OF RAT *NEU*-TRANSFORMED NIH 3T3 CELLS, Mien-Chie Hung and Dean B. Reardon, Department of Tumor Biology, The University of Texas M. D. Anderson Cancer Center,1515 Holcombe Blvd., Houston, Texas 77030

The c-*neu* gene encodes for a transmembrane tyrosine kinase growth factor receptor which is related to but distinct from the EGF receptor. Little information is known concerning the protein factors involved in c-*neu* specific signal transduction following p185 receptor activation. Using the rat c-*neu* oncogene as a model system, a mutagenic assay system has been developed to obtain morphologically non-transformed revertants of the B104-1-1 cell line (NIH 3T3 cells transformed with the genomic rat c-*neu* oncogene). These revertants, designated *neu*-R1 and *neu*-R2, have an ordered growth pattern, display contact inhibition, are unable to pile up at random, and are morphologically similar to NIH 3T3 cells. Doubling times for *neu*-R1 and *neu*-R2 are slightly greater than that of NIH 3T3 cells and twice that of the B104-1-1 parental cell line. Unlike the B104-1-1 parental cell line, *neu*-R1 and *neu*-R2 are unable to form colonies in soft agar, produce foci on a growing cell monolayer, or grow in medium containing less than five percent calf serum. Both *neu*-R1 and *neu*-R2 still express the c-*neu* p185 protein product at levels sufficient to induce cellular transformation in the NIH 3T3 model system. However, phosphotyrosine levels of *neu* in these revertants is highly reduced as compared to activated *neu* in B104-1-1 cells and is comparable to the level seen for the endogenous non-activated p185 protein product in NIH 3T3 cells. This indicates that the p185 protein product exists in a non activated form in the revertant cell lines. In addition, when *neu*-R2 and NIH 3T3 cells are stably transfected with c-*neu*, H-*ras*, N-*ras*, v-*mos*, v-*abl*, or v-*fos* oncogenic expression constructs, focus forming efficiency for *neu* -R2 *is twenty fold* reduced when compared to NIH 3T3 focus formation. Most surprisingly, *neu*-R1 is completely resistant to transformation/focus formation even when transfected with high levels of the above oncogenic expression constructs. This suggests that both revertant cell lines contain defects in downstream signalling pathways which prevent transmission of the necessary signals required for cellular transformation. Furthermore, p185 phosphotyrosine appears to be required for the proper transmission and activation of signals necessary for *neu* induced cellular transfomation.

BZ 121 ROLE OF PROTEIN KINASES IN MAMMARY EPITHELIAL CELL DIFFERENTIATION, Nancy Hynes, Barbara Marte and Daniela Taverna, Friedrich Miescher Institute, P.O. Box 2543, 4002 Basel, Switzerland.

HC11 cells are normal mouse mammary epithelial cells which have retained important characteristics of mammary differentiation. Treatment of the cells with the lactogenic hormone mix, dexamethasone, insulin and prolactin (DIP), leads to the production of beta-casein, one of the major milk proteins. The hormones increase transcription from the beta-casein gene promoter. The HC11 cells express different members of the receptor tyrosine kinase family including EGF, erbB-2, insulin, IGF-1, PDGF, and basic FGF receptors. We have studied the effects which activation of each receptor has upon the differentiation process. Growth of HC11 cells in EGF and basic FGF allows them to become competent to respond to the subsequent addition of lactogenic hormones and synthesize high levels of beta-casein. Cells expressing an activated erbB-2 receptor (introduced by transfection) are also competent to respond to the DIP mix. In contrast, cells grown in insulin or PDGF do not synthesize beta-casein in response to the lactogenic hormones. During the growth of the cells activation of each receptor tyrosine kinase has distinct effects upon the differentiation process suggesting that the receptors couple to partially overlapping but distinct signaling pathways. Specific substrates implicated in signaling from receptor tyrosine kinases are now being examined for their activity following addition of the individual growth factors. During the actual differentiation process the activation of the glucocorticoid, insulin and prolactin receptors is essential for beta-casein productio. None of the other growth factors, with the exception of IGF-1, can substitute for insulin in the lactogenic hormone mix. The prolactin receptor, a member of the cytokine receptor family, does not display kinase activity, but may activate intracellular kinases. It has been shown that PK-C can be activated by prolactin addition to rat liver cells. When selective PK-C inhibitors are included in the DIP mix there is an inhibition of transcription from the beta-casein gene promoter. These results suggest that in HC11 cells PK-C is required for DIP induced casein production and that prolactin receptor signals through PK-C. We have examined the isoforms of PK-C expressed in the HC11 cells and are attempting to verify PK-C activation upon prolactin addition to the cells.

BZ 122 GERMLINE TARGETING OF THE MOUSE *trk*B GENE Rüdiger Klein[1], Wolfgang Wurst[2], Linda K. Long[1], Alexandra L. Joyner[2], and Mariano Barbacid[1]. [1]Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543. [2]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada.

The Trk family of tyrosine protein kinases have been recently identified as the functional receptors for the NGF family of neurotrophins which include NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). NGF binds to and activates gp140^{trk} receptors. NT-3 primarily activates gp145^{trkC} receptors, although in certain cells it can also show limited activity towards gp140^{trk} and gp145^{trkB}. Interestingly, the gp145^{trkB} tyrosine kinase serves as a receptor for two different neurotrophins, BDNF and NT-4/5. Transcripts encoding gp145^{trkB} have been identified in most structures of the CNS as well as in a wide variety of peripheral neurons. In addition, the *trk*B locus encodes a second receptor that lacks the kinase domain. Expression of this non-catalytic receptor appears to be restricted to the choroid plexus and the ependymal layers of the cerebral ventricles. In order to understand the role of the *trk*B receptor in the development and maintenance of the mammalian nervous system, we have targeted the *trk*B locus in embryonic stem (ES) cells. A *trk*B replacement plasmid was constructed in which sequences from the second exon of the tyrosine kinase domain were replaced by the *neo* gene under control of the mouse PGK-1 promoter. This vector also carries a HSV thymidine kinase gene to provide for negative selection with gancyclovir (GANC). The frequency of homologous recombination as determined by PCR analysis was about 1 in 90 G418^R, GANC^R colonies. Upon verification by Southern analysis of genomic DNA, C57Bl/6 blastocysts were injected with targeted ES cell clones and implanted into foster CD1 females. Chimeric offspring with >50% ES cell contribution as judged by Agouti coat color were found to transmit the disrupted *trk*B allele through their germline. The heterozygous offspring appear normal at 6 weeks of age. They are currently being mated to produce mice homozygous for the *trk*B mutation.

BZ 123 ACTIVATION OF PI 3-KINASE BY INSULIN IN 3T3L1 ADIPOCYTES, Lou Lamphere, Christopher L. Carpenter*, Lewis C. Cantley*, and Gustav E. Lienhard, Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755 and *Department of Physiology, Tufts University School of Medicine, Boston MA 02114

Insulin activation of PI3 kinase in 3T3-L1 adipocytes was examined. The activity of the enzyme was determined in an *in vitro* assay by the formation of ^{32}P-labeled PIP_3 from PIP_2 and ^{32}P-γ-ATP when added to the cytosol of basal and insulin treated cells. Insulin resulted in a 2 to 15 fold increase in PI 3-kinase activity. The effect of insulin was maximal after exposure of cells for 1 min and remained constant for 60 min. The concentration of insulin required for half maximal stimulation was about 5 nM.

A 160 kDa protein, known as Insulin Receptor Substrate 1 (IRS1), is rapidly phosphorylated on Tyr in response to insulin. Previously, we have provided qualitative evidence that in 3T3-L1 adipocytes the P-Tyr form of IRS1 associates with PI 3-kinase via the SH2 domains of the latter. We have further examined the interaction of PI 3-kinase with IRS1. IRS1 in the cytosol from basal and insulin-treated cells was immunoprecipitated with antibodies against the C-terminal peptide of IRS1. The original cytosol and the depleted cytosols (cytosols after absorption) were then immunoblotted for IRS1 and for PI 3-kinase (85 kDa subunit). The results showed that greater than 50% of the PI 3-kinase co-immunoprecipitated with IRS1 in the cytosol from insulin treated cells, but not in that from basal cells. These findings indicate that insulin activation of PI 3-kinase results from the binding of the insulin-elicited P-Tyr form of IRS1 to this enzyme.

BZ 124 MOLECULAR ANALYSIS OF THE LIGAND BINDING SITE OF THE KIT RECEPTOR. Sima Lev, Janna Blechman, David Givol and Yosef Yarden. Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel.

Binding of the stem cell factor (SCF) to the kit- receptor tyrosine kinase stimulates a variety of biochemical responses that culminated in cellular prolifaration, migration or survival. The extracellular domain of Kit consists of five immunoglobulin (Ig)-like domains. To localize the ligand binding site of the Kit receptor we utilized two experimental approaches: First, analysis of the binding properties of soluble proteins that included two or three amino-terminal Ig-like domains, or the entire extracellular portion (Kit-X). The second approach involved construction and binding analysis of chimeric human-mouse Kit-receptor molecules and was based on the fact that human SCF has much higher affinity to the human receptor as compared to the murine Kit. These approaches were complemented by the use of ligand-competitory monoclonal antibodies to Kit, and collectively indicated that the binding site is confined to the N-terminal half of the ectodomain. Moreover, we concluded that the second Ig-like domain confers human-SCF binding specificity and contains the major determinants of ligand recognition, but its flunking Ig-like domains also contribute to high affinity binding of SCF.

BZ 125 THE ROLE OF p21ras IN THE TORSO SIGNALING PATHWAY, Xiangyi Lu, Nidhi Williams, Tom Roberts & Norbert Perrimon, Dept. of Genetics, Harvard Medical School, 200 Longwood Ave, Boston, MA 02115

Terminal structures of Drosophila embryos are specified by localized activation of the torso (tor) receptor tyrosine kinase (RTK) at the embryonic poles. Generalized activation of the receptor represses segment formation in the central region. We found that expression of an activated D-ras1 gene maternally by heat shock produces similar defects. Moreover, the mammalian oncogene v-ras rescues terminal structures absent in embryos from tor null mother when the v-ras protein is injected into the embryos posteriorly. The rescue by v-ras requires D-raf activity maternally, suggesting that $p21^{ras}$ is a critical mediator between tor RTK and D-raf serine/threonine kinase. Conversely, injection of dominant negative ras^{N17} protein partially block tor signaling in wild type embryos as visualized by flz expression. Consistent with the proposed role of csw in tor signaling pathway elucidated genetically, v-ras also rescue the maternal effects of csw, a protein tyrosine phosphatase.

BZ 126 Biological profile of CGP 52411: a tyrosine protein kinase inhibitor with selectivity for the EGF-receptor enzyme family

Lydon N. B., Buchdunger E., Mett H., Regenass U., Muller M., Trinks U.and Traxler P. Ciba-Geigy Limited., Oncology and Virology Research Department, CH-4002 Basel, Switzerland.

Enhanced tyrosine protein kinase (TPK) activity has been associated with proliferative diseases such as cancer, atherosclerosis and psoriasis. The role of TPKs in cellular proliferation suggest that TPK inhibitors could have therapeutic potential in neoplastic and non-malignant proliferative diseases. The EGF-R kinase family of transmembrane growth factor receptors, which are involved in epithelial proliferation in response to mitogenic growth factors, have been strongly implicated in malignant tumor growth. In the present poster we describe the biological profile of CGP 52411, a selective tyrosine protein kinase inhibitor of the EGF-R family of kinases.

CGP 52411 represents a selective inhibitor of the EGF-R tyrosine protein kinase. *In vitro* CGP 52411 selectively inhibited the TPK activity of the EGF-R (IC_{50} of 0.3-0.8 μM) and receptor autophosphorylation (IC_{50} of 1 μM). In cells, CGP 52411 selectively inhibited EGF dependent receptor autophosphorylation (IC_{50} 1-10 μM), EGF stimulated cellular tyrosine phosphorylation (IC_{50} 3.4-6.7μM) and $p185^{c\text{-}erbB2}$ autophosphorylation (IC_{50} of 10 μM). Ligand induced receptor autophosphorylation of the PDGF-R was not effected by up to 100 μM CGP 52411. CGP 52411 was also able to selectively block c-*fos* induction by EGF, but not by PDGF, FGF and PMA. CGP 52411 inhibited proliferation of the EGF dependent MK cell line and the T24 bladder carcinoma lines with IC_{50} values which are similar to those determined for inhibition of autophosphorylation. The MTD in female BALB/c mice for a single administration of CGP 52411 was >500 mg/kg when given orally and 250 mg/kg when given i.p. Antitumor experiments were performed with both routes of application. Applications of 1/10 to 1/80 of the MTD gave dose dependent anti-tumor activity. The maximum response achieved was a T/C (treatment/control) value of 20% with 25 mg / kg i.p. and 50 mg / kg p.o. respectively.

BZ 127 IDENTIFICATION OF A NOVEL PUTATIVE TYROSINE KINASE FROM A MOUSE NEUROBLASTOMA CELL LINE, NB 41

M. Leia D. Maminta, Korwyn L. Williams and Thomas F. Deuel. Hematology Research Dept., Jewish Hospital of St. Louis at Washington University Medical Center, St. Louis, MO 63110

A number of growth factor receptors have tyrosine kinase activities that are important for the signalling processes and regulation of cellular events. To identify novel growth factor receptors, we used primers designed from the highly conserved tyrosine kinase domains for PCR amplification of cDNA of the mouse neuroblastoma cell line, NB41. The amplified fragment of about 230 bp was cloned and sequenced. Comparison of the sequences with the Genebank showed that most of the clones were homologous to the basic FGF family of receptors. However, a novel clone, designated NBtk-1, is related to the *met* proto-oncogene family of tyrosine kinases. By Northern analysis, using the NBtk-1 fragment as a probe, we identified two transcripts from NB41 and NIH3T3 cell lines. One transcript is 2.6 kb, while a shorter transcript is about 2.1 kb, which may indicate differential splicing of the NBtk-1 mRNA. We will further study the significance of the difference in transcript sizes. The same transcripts are also present in mouse mammary cell lines, MMT and MM3MG. In contrast, we also found that in human mammary cell lines, MCF-7 and T47D, the NBtk-1 probe hybridizes to a slightly longer message of about 2.8 kb. We have used this probe to screen an NB41 cDNA library for further study of this novel tyrosine kinase.

BZ 128 EXPRESSION OF FUNCTIONAL PLATELET-DERIVED GROWTH FACTOR RECEPTORS ON HUMAN NEUROBLASTOMA CELLS, Toshimitsu Matsui, Tatsuo Tsukamoto, Mitsuhiro Ito, and Kazuo Chihara, Third division, Department of Medicine, Kobe University School of medicine, Kobe 650, Japan

Both platelet-derived growth factor (PDGF) A and B chains are expressed in mammalian neurons and are thought to contribute to the development of the nervous system. However, their precise roles still remain to be clarified. Recently, two PDGF receptor genes whose products show different affinities for the three PDGF isoforms were identified, and designated as α and β PDGF receptors. Each receptor can independently undergo tyrosine autophosphorylation in response to the specific PDGF isoforms, and couple with the signaling pathways for a variety of biological responses. Thus, the presence of specific PDGF isoforms and the differential expression of each PDGF receptor gene product are thought to be major determinants of the spectrum of known responses of cells to PDGF. In the present studies, we examined the expression of two PDGF receptor genes in human tumor cell lines derived from neural crest. The expression of α and β PDGF receptors were detected in a wide variety of neural crest-derived human tumor cell lines such as neuroblastoma, primitive neuroectodermal tumor and Ewing's sarcoma by RNA blot analysis, and confirmed by immunoblot analysis. We also demonstrated the PDGF receptors on the human neuroblastoma cell lines were biologically functional. The chemotactic as well as mitogenic activities were induced by either PDGF-AA or PDGF-BB in serum-free medium. These biological activities of PDGF isoforms were accompanied by morphological changes showing neuronal cell maturation. Moreover, PDGF coordinately increased the levels of the transcript of the mid-size neurofilament gene. These findings suggest that PDGF isoforms are involved not only in the promotion of the neuroblastoma cell growth, but also in neuronal cell migration, growth and differentiation in human brain development.

BZ 129 FUSION OF V-ERB B WITH THE ESTROGEN RECEPTOR PRODUCES A HORMONE DEPENDENT CHIMERA FOR ANALYSIS OF CELLULAR TRANSFORMATION.

Deborah J. McCarley, Randall C. Schatzman, and Martin McMahon*. Institute of Biochemistry and Cell Biology, Syntex Research, Palo Alto, CA 94304. *DNAX Research Institute, 901 California Avenue,Palo Alto, CA 94304.

The v-erb-B oncogene arose from the transduction of epidermal growth factor receptor (ERF-R) sequences into a retroviral genome. Loss of the EGF binding domain and a carboxy-terminal truncation of the EGF-R led to constitutive activation of the transforming ability of the v-erb-B gene product in the absence of hormone. In order to examine the mechanism by which v-erb-B modulates transformation, a conditional form of the v-erb-B oncogene was constructed. The fusion of sequences of the hormone binding domain of the human estrogen receptor to the carboxy terminus of the v-erb-B oncogene renders transformation by v-erb-BER dependent on the presence of estradiol. Initially we characterized the protein product of the v-erb-BER construct to examine whether it could be utilized to facilitate analysis of the mediators of v-erb-B transformation. The addition of estradiol to v-erb-BER/3T3 cells caused an increase in the phosphotyrosine content of the v-erb-BER protein by one hour, peaking at 4 to 6 hours. The phosphotyrosine content of other proteins also increased during this time course. There was a 2-fold increase in the amount of v-erb-BER protein when estradiol was added to the cells. The specific protein kinase activity as measured by both autophosphorylation and transphosphorylation increased 4 to 6 fold. The half-life was approximatly thirty minutes whether or not estradiol was present. This is significantly shorter than the half life of the wild type v-erb-B protein. The v-erb-BER protein is glycosylated similar to wild type v-erb-B as determined by tunicamycin treatment and is localized to the membrane fraction of the cells. Transformation of 3T3 cells by both wild type v-erb-B and v-erb-BER in the presence of estradiol resulted in the tyrosine phosphorylation of PLC-γ, p120, a protein which becomes phosphorylated on tyrosine in response to EGF or v-src transformation of 3T3 cells shows no increase in tyrosine phosphorylation in 3T3 cells transformed by wild type v-erb-B or v-erb-BER. Raf-1 protein is modulated in wild type v-erb-B transformed cells; we are in the process of determining whether transformation of v-erb-BER/3T3 cells in the presence of estradiol modulates the Raf-1 protein.

BZ 130 EXTRACELLULAR PROTEOLYTIC CLEAVAGE BY UROKINASE IS REQUIRED FOR THE ACTIVATION OF HEPATOCYTE GROWTH FACTOR/SCATTER FACTOR, Luigi Naldini, Luca Tamagnone, Elisa Vigna, and Paolo M. Comoglio, Department of Biomedical Sciences and Oncology, University of Torino Medical School, 10126, Torino, Italy

Hepatocyte Growth Factor/Scatter Factor (HGF/SF) is a product of stromal fibroblasts which induces motility, invasion and growth of epithelial and endothelial cells. HGF/SF is the ligand for the receptor tyrosine kinase encoded by the *MET* proto-oncogene. We found that, unlike most of the known growth factors, HGF/SF was secreted by producer cells as a precursor devoid of biological activity (pro-HGF/SF). Maturation of this precursor into the bioactive molecule took place in the extracellular environment by a serum-dependent proteolytic cleavage. We also identified the extracellular protease competent for the activation of pro-HGF/SF with the urokinase-type plasminogen activator (uPA). This was shown by both *in vitro* assays with the pure proteins and the use of specific uPA inhibitors on cultured cells. UPA is a well known mediator of matrix invasion in morphogenesis and tumor metastasis. Its proposed role as a pro-HGF/SF convertase suggests that some of the growth and invasive cellular responses mediated by this enzyme may involve activation of HGF/SF.

BZ 131 INTERACTIONS OF THE CSK SH2 DOMAIN WITH TYROSINE PHOSPHORYLATED PROTEINS.

Kellie Neet and Tony Hunter, Molecular Biology and Virology Laboratory, Salk Institute for Biological Studies, P.O. Box 85800, San Diego, CA 92186-58000

C-terminal Src Kinase (CSK) is a ubiquitous protein tyrosine kinase that phosphorylates pp60$^{c\text{-}src}$ on tyrosine 527, suppressing pp60$^{c\text{-}src}$ catalytic activity. CSK contains an SH2 domain and an SH3 domain and lacks an N-terminal myristylation signal. SH2 domains have been shown to bind to phosphotyrosine-containing sequences and SH3 domains are believed to bind cytoskeletal elements. We are analyzing the mechanism by which CSK, a putative cytosolic enzyme, recognizes and phosphorylates pp60$^{c\text{-}src}$, a membrane-associated protein. We have found that the SH2 domain of CSK, when expressed in bacteria as a glutathione S-transferase fusion protein, will associate with activated PDGF Receptor (PDGFR) and several other tyrosine phosphorylated proteins. The association is dependent upon ligand-induced receptor activation and phosphorylation. Since pp60$^{c\text{-}src}$ associates with and becomes activated by the PDGFR, it is possible that CSK recognizes pp60$^{c\text{-}src}$ by also binding to a PDGFR complex. We have raised a polyclonal rabbit antisera against a synthetic peptide corresponding to the C-terminus of CSK. We are currently investigating the *in vivo* interactions between CSK and the PDGFR and are further characterizing the nature of the *in vitro* interaction between the CSK SH2 domain and the PDGFR.

BZ 132 *Abstract Withdrawn*

BZ 133 **Selective Inhibition of the EGF and Neu receptors by Tyrphostins**

Nir Osherov[1], Aviv Gazit[1], Chaim Gilon[2], and Alexander Levitzki[1]

[1]Department of Biological Chemistry, Institute Of Life Sciences, Hebrew University of Jerusalem, Israel. [2]Department of Organic Chemistry, Institute of Life Sciences, Hebrew University of Jerusalem, Israel.

The HER2 (neu/erb-B2) proto-oncogene codes for a 185kd transmembrane receptor with tyrosine-kinase activity and with high homology to the EGF-receptor (HER1). It is capable of transforming 3T3 cells when overexpressed and is amplified in approximately 30% of human breast and ovary carcinomas. Direct correlation has been shown between the degree of amplification of the gene and its expression with the prognosis of the disease in patients.

These findings prompted us to develop protein tyrosine kinase inhibitors (tyrphostins) which selectively inhibit the HER2 kinase. Two groups of tyrphostins were developed: one which is highly selective in inhibiting HER1 as opposed to HER2 kinase activity, the other is highly selective in inhibiting HER2 as compared to HER1 kinase activity.

The most promising of these compounds were subsequently tested for their inhibitory activity in intact 3T3 cells transfected with HER1 or HER2. Surprisingly, no preferential inhibition by these compounds in intact cells was observed in three different assays.

The paradox between the results of the cell-free as compared to intact cell assays was further explored and elucidated: These compounds were shown to be competitive with ATP and preferential inhibition was restored in ATP-depleted cells. On the basis of our findings we propose that the high levels of ATP in intact cells are blocking inhibitor binding to the receptor kinase ATP-binding site indicating that the in-vivo inhibition of [^{3}H]thymidine uptake seen with these compounds may be at least partially due to effects downstream of the receptors. Our results are also relevant in understanding the mode of action of several other tyrosine kinase blockers such as genistein, orobol and Lavendustin A which are also competitive with ATP.

BZ 134 RAS ACTIVATION BY MUTANT INSULIN RECEPTORS, D.M. Ouwens[1], G.J. Pronk[2], A.P.R.M. Osterop[1], G.C.M. Van der Zon[1], W. Möller[1], and J.A. Maassen[1], [1]Department of Medical Biochemistry, Sylvius Laboratory, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands, and [2]Laboratory for Physiological Chemistry, University of Utrecht, Vondellaan 24A, 3521 GG Utrecht, The Netherlands.

Insulin stimulation of cells expressing elevated numbers of insulin receptors (IR) results in a rapid conversion of Ras.GDP into Ras.GTP (Burgering et al., 1991, EMBO J. 10: 1103-1109). The IR is mainly autophosphorylated on three tyrosine residues. To investigate the role of the individual tyrosine residues in Ras-activation, various IR mutants, in which the three major tyrosine autophosphorylation sites were stepwise substituted by phenylalanines, were expressed in CHO cells (expression vectors were kindly provided by dr. R.A. Roth). The cells were stimulated with insulin and examined for Ras.GTP formation, and association of the Ras GTPase activating protein (GAP) to the phosphorylated IR. In addition, we determined mitogenic activation. It was found that an IR mutant, in which tyrosines 1158, 1162, and 1163 were replaced by phenylalanines (FFF), did not activate Ras. A mutant IR, in which tyrosine 1158 was substituted by phenylalanine (FYY), was however capable of Ras activation. Further, immunoprecipitated FFF-receptors associated only weakly to the SH2-SH3 domain of recombinant GAP in vitro, whereas a strong signal was obtained in the case of wild type and FYY receptors. These results suggest that tyrosines 1162 and 1163 are implicated in both Ras-activation and GAP-association. Data on mitogenic signaling and Ras activation of these and other IR mutants will be presented.

BZ 135 EXPRESSION OF HETEROLOGOUS RECEPTORS AND THEIR LIGANDS IN FACTOR DEPENDENT CELLS, Robert E. Pacifici, Victoria J. Costigan and Arlen Thomason, Department of Molecular Biology, Amgen Inc., Amgen Center, Thousand Oaks, CA 91320

Recent advances in PCR technology have facilitated the discovery of many novel growth factor receptors. Identification of their cognate ligands is complicated by their low abundance in tissues or cultured cells. For possible use in expression cloning of receptor ligands, we have utilized factor dependent cell lines to select for autocrine cells which express the receptor of interest and its ligand. 32D is a murine cell line which is dependent on IL3 for growth. A retroviral expression vector containing heterologous receptor constructs was introduced into 32D cells by electroporation, and G418 was used to select stable transfectants. G418 resistant pools were sorted by flow cytometry to yield a >95% receptor positive population. This strategy was utilized to introduce several receptor tyrosine kinases (including EGF-R), a cytokine receptor (EPO-R), as well as several receptor chimeras. Expression of introduced receptors was confirmed by western blot analysis. 32D cells expressing EGF-R or EPO-R respond to EGF or EPO (respectively) in [^{3}H]thymidine uptake and cell proliferation assays. Receptor down regulation in response to EGF has been demonstrated in 32D-EGF-R cells. These cells can be routinely passaged in EGF or EPO, in the absence of IL3 while maintaining their factor dependent phenotype. 32D-EPO-R cells were retransfected with another expression vector containing EPO cDNA. Cells were selected based on their ability to grow in the absence of IL3. Positive transfectant pools produce measurable quantities of EPO, and their growth is severely inhibited (>75%) by neutralizing EPO antibodies. These data indicate that an EPO/EPO-R autocrine loop has been established which maintains independent growth in these cells.

BZ 136 ANALYSIS OF DIFFERENT FORMS OF tie, AN ENDOTHELIAL CELL RECEPTOR TYROSINE KINASE, Juha Partanen, Elina Armstrong, Tomi P. Mäkelä, Jaana Korhonen, Minna Sandberg[1], Risto Renkonen[2], Sakari Knuutila[3], Kay Huebner[4] and Kari Alitalo. Cancer Biology Laboratory, Departments of Virology and Pathology, Departments of of Bacteriology[2] and Genetics[3], University of Helsinki, Haartmaninк. 3, 00290 Helsinki 29, FINLAND, Department of Medical Biochemistry[1], University of Turku, 20520 Turku, FINLAND, The Fels Institute for Cancer Research and Molecular Biology[4], Temple University School of Medicine, Philadelphia, PA 19 140,USA

We describe cloning and characterization of *tie*, a novel type of human endothelial cell surface receptor tyrosine kinase. The extracellular domain of the predicted *tie* protein product has an exceptional multidomain structure consisting of a cluster of three epidermal growth factor (EGF) homology motifs embedded between two immunoglobulin-like loops followed by three fibronectin type III repeats next to the transmembrane region. Additionally, alternative splicing creates different *tie*-type receptors. A cDNA form lacking the first of the three EGF homology domains was isolated as well as a form encoding a receptor without the tyrosine kinase domain. Cells transfected with *tie* cDNA expression vector produce glycosylated polypeptides of 117 kD reactive to antisera raised against the *tie* carboxy terminus. The *tie* protein could be immunoprecipitated with antiphosphotyrosine antibodies, supporting the prediction that *tie* is a tyrosine specific kinase. The *tie* gene was localized to chromosomal region 1p33-34. High amounts of *tie* mRNA were detected in endothelial cell lines and in some myeloid leukemia cell lines with erythroid and megakaryoblastoid characteristics. mRNA *in situ* studies further indicated the endothelial expression of the *tie* gene. The *tie* receptor tyrosine kinase may have evolved for multiple protein-protein interactions, possibly involving cell adhesion to the vascular endothelium. Our ongoing work is concentrated on the identification of the growth factor binding to the *tie* receptor.

BZ 137 CELL-TYPE SPECIFIC INTERACTION OF NEU DIFFERENTIATION FACTOR (NDF/HEREGULIN) WITH NEU/HER-2, Elior Peles[1], Eldad Tzahar[1],Rachel Ben Levy[1], Duanzhi Wen[2] and Yosef Yarden[1].
[1]Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel and [2]Amgen Center, Thousand Oaks, California 91230

The Neu/HER-2 receptor tyrosine kinase is overexpressed in human adenocarcinomas of the breast and the ovary. A 44-kilodalton glycoprotein that elevates tyrosine phosphorylation of Neu has been isolated and named Neu differentiation factor (NDF) or heregulin. This factor was able to form covalent complexes with p185^{neu} upon chemical cross-linking. To further examine the possibility that NDF is a direct ligand of p185^{neu} we surveyed its interaction with a series of cell lines. Here we show that recombinant NDF affects tyrosine phosphorylation of Neu in human tumor cells from breast, colon and neuronal origins, but not in ovarian cells that overexpress the receptor. Nevertheless p185^{neu} molecules from ovary and mammary cells are biochemically and immunologically indistinguishable. Similarly, *neu*-transfected ovary cells, and also fibroblasts that ectopically overexpress p185^{neu}, did not respond to NDF. Nevertheless, unlike breast-derived Neu, the ovarian protein did not display covalent cross-linking to radiolabeled NDF and was devoid of ligand-induced association with phosphatidylinositol 3'-kinase. Taken together, our results raise the possibility that the interaction of NDF with Neu involves an additional cellular component whose identity is still unknown, but its tissue distribution is more restricted than the expression of the *neu* gene.

BZ 138 EXPRESSION OF A DOMINANT INHIBITORY RAS MUTANT BLOCKS THE Shc-INDUCED PC12 DIFFERENTIATION. Giuliana Pelicci*, Anna E. Salcini*, Luisa Lanfrancone*, Simona Mele*, Enrica Migliaccio*, Sergio Giuli*, Joan Brugge*, Joseph Schlessinger§, M. Rozakis-Adcock, Jane McGlade, G. Mbamalu, Tony Pawson and P.G. Pelicci*, *Ist. Clinica Medica, Perugia University, Perugia 06100; Italy; §Dept. Pharmacology, NYU Medical Center, New York, NY 10016; Ariad Pharmaceuticals Inc., Cambridge, Mass. 02139; Dpt. Molecular and Medical Genetics, Toronto Univ., M5G 1X5 Canada.

We recently isolated a new SH2-containing sequence, SHC. The SHC locus is split into 15 exons and encodes 3 isoforms (p46, p52, p66) that contain a C-terminal SH2 domain, an adjacent gly/pro rich motif and differ at their N-termini. A number of findings suggest that Shc proteins are biologically relevant substrates of oncogenic tyrosine kinases (TK) and are involved in controlling the Ras signalling pathway: i) are widely expressed; ii) are rapidly phosphorylated on tyrosine in response to activation of a variety of growth factor receptors (EGF, PDGF, NGF, CSF-1, GM-CSF, MET, ERB-B2); iii) are phosphorylated by cytoplasmic TK; iv) form stable complexes with the Grb-2/sem-5 gene product, which is implicated in Ras activation in *C. elegans* and mammalian cells; v) when overexpressed induce transformation of rodent fibroblasts; vi) induce fibroblasts to transit the G1 phase of the cell cycle in the absence of growth factors; vii) tyrosine-phosphorylation appears to be essential for their biological activity (e.g. transformation). To test the possibility that Shc proteins are involved in the control of Ras signalling pathway we analyzed the effect of the expression of a dominant inhibitory Ras mutant on the biological activity of Shc. Overexpression of the shc cDNA in PC12 cells induced neurite outgrowth and this effect was blocked by expression of a dominant inhibitory H-Ras mutant, RasS17N.

BZ 139 EGF OR PDGF INTERRUPT INTERCELLULAR COMMUNICATION IN THE ABSENCE OF RECEPTOR TYROSINE KINASE ACTIVITY, Diane B. Pelletier and Alton L. Boynton, Department of Cell and Molecular Biology, Pacific Northwest Research Foundation, Seattle, WA, 98122

EGF and PDGF bind to membrane bound receptors resulting in rapid activation of intrinsic receptor tyrosine protein kinase (TPK) which stimulates autophosphorylation and phosphorylation of cellular substrates. Addition of either PDGF or EGF to confluent, proliferatively quiescent C3H10T1/2 mouse fibroblasts interrupts intercellular communication (ICC), which occurs via gap junction proteins termed connexins. ICC appears to be regulated by the serine or tyrosine phosphorylation and/or dephosphorylation of one or more members of the connexin protein family. Interruption of ICC is correlated with mitogen-activated cell proliferation and neoplastic transformation. We have tested whether interruption of ICC by either PDGF or EGF occurs in the absence of receptor TPK activity in two models systems: inhibition of the PDGF receptor TPK by the isoflavone genistein and transmodulation of the EGF receptor TPK by PDGF pre-treatment. ICC was measured by microinjection of Lucifer yellow into a single cell and passage of this fluorescent dye through gap junctions. In both models, TPK activity was blocked as determined by anti-phosphotyrosine immunoblots of the receptors and their substrates. EGF or PDGF interrupted ICC in receptor TPK-inactive cells to the same degree as cells with active receptor TPK. Thus, PDGF and EGF receptor-mediated inhibition of ICC can be dissociated from detectable receptor TPK activity. Regulation of ICC by PDGF or EGF may occur via receptor TPK-independent signal transduction.

BZ 140 TYROSINE PROTEIN KINASE INHIBITORS, TYRPHOSTINS, INHIBIT SOME CELLULAR EVENTS IMPLICATED IN SIGNAL TRANSDUCTION, J. Pierre, S. Piperno, W. Agbotounou, S. Mousset, M. Pierre* and A. Jacquemin-Sablon , U140 INSERM and URA 147 CNRS, Institut Gustave Roussy, 94800 Villejuif, France; *U96 INSERM, Hôpital de Bicêtre, 94270 Le Kremlin-Bicêtre France.

We have examined the effect of tyrphostin (RG 50864), an inhibitor of EGF-Receptor tyrosine kinase on cellular events mediated by EGF and TPA in NIH3T3 cells.

Treatment of EGF stimulated cells with tyrphostin reduced the level of c-fos and c-myc transcription. The same observation was done, when the cells were stimulated by TPA in the presence of tyrphostins. This suggests that a common step in EGF and TPA signal transduction pathways leading to an increased expression of the protooncogenes, is inhibited by tyrphostin. The MAP kinase, which is activated by phosphorylation on tyrosine and threonine after EGF and TPA stimulation could be this step. The MAP kinase activity was inhibited when the cells are treated with increasing tyrphostin concentrations, reducing the level of the MAP kinase activity to 15 % of the level observed in the control cells. The MAP kinase extracted from cells stimulated by EGF and TPA was not inhibited in vitro by tyrphostins. These results indicate that tyrphostin inhibits activation of the MAP kinase. The inhibitory effect of tyrphostins of the MAP kinase could participate to the inhibition of the mitogenic effect of the EGF and TPA in NIH3T3 cells.

BZ 141 STRUCTURE AND FUNCTION OF HER3/c-*erb*B-3: STUDIES USING SITE-DIRECTED MUTAGENESIS, Sally A. Prigent and William J. Gullick, ICRF Oncology Group, Hammersmith Hospital, Du Cane Road, London W12 ONN.

HER3 is a recently described member of the EGF receptor family for which no ligand has been identified. Despite homology and shared sequence identity with EGF receptor and HER2, the primary amino acid sequence of HER3 contains amino acid replacements at two potentially critical sites within the kinase domain (His-740, Asn-815) which are conserved in all other known serine/threonine and tyrosine kinases as glutamate and aspartate respectively. Using transfected human fibroblasts as a source of HER3 protein we have been unable to demonstrate basal kinase activity by immune-complex kinase assays, immunoblotting of total cell lysates with antiphosphotyrosine antibodies or by kinase assays on membrane preparations. This suggests the possibility that HER3 may represent a novel class of growth factor receptors with an inactive kinase. To address this question, mutants have been constructed in which His-740 and Asn-815 have been mutated to Glu and Asp respectively to determine whether the kinase could be activated. The effect of ligand stimulation is being investigated by the construction and expression of chimaeric genes containing the extracellular portion of EGF receptor linked to the intracellular domain of HER3 to examine whether kinase activity can be stimulated by EGF.

BZ 142 OVEREXPRESSION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR IS ACCOMPANIED WITH AN INCREASED ACTIVITY OF c-src OR c-src-ASSOCIATED TYROSINE KINASES, Gert Rijksen, Astrid E. Ottenhoff-Kalff, Marijke P. Vendrig and Gerard E.J. Staal, Department of Hematology, Laboratory of Medical Enzymology, University Hospital, P.O. Box 85.500, 3508 GA Utrecht, The Netherlands

In breast cancer and several other human tumors overexpression of the epidermal growth factor receptor (EGFr) is a negative prognostic indicator, suggesting that it may be important for the pathogenesis of the disease. In recent studies we showed that protein tyrosine kinase (PTK) activity in breast cancer is consistently increased and correlates with an unfavorable prognosis. Immunoprecipitation studies using anti-src antibodies identified the majority of PTK as c-src or c-src associated PTK's. To determine whether overexpression of EGFr and activation of cytosolic c-src are related, we studied both phenomena in two breast cancer cell lines derived from ZR 75-1 cells. In one of these cell lines (ZR 11) functional EGFr was overexpressed 60-times above the parental cell line as well as the control cell line (ZR 9B11) in which only vector was transfected (Valverius et al., Int.J.Cancer 46, 1990, 712-718). The growth characteristics of both cell lines were comparable. In the absence of serum-derived growth factors the addition of exogenous EGF stimulated cell growth 2-3 fold in both cell lines. c-Src was immunoprecipitated from the cytosolic fractions using Ab 327 and analysed by immunoblotting with antibodies LA 074, directed against the N-terminal part of the protein, and CST-1 (donated by Dr. S.A. Courtneidge), which recognizes the C-terminus. The results revealed comparable amounts of full size, intact c-src protein in cytosolic and membrane fractions of both cell lines. However, the amount of precipitable PTK activity from the cytosolic fraction was increased 1.5 - 2 fold in ZR 11 compared to ZR 9B11, suggesting that the overexpression of EGFr results in an activation of c-src or other PTK's which may be associated with c-src.

BZ 143 LIGAND-INDUCED AUTOPHOSPHORYLATION OF THE PDGF ß-RECEPTOR: MAPPING AND ANALYSIS OF AUTO-PHOSPHORYLATION SITES IN THE JUXTAMEMBRANE DOMAIN AND IN THE TAIL

Lars Rönnstrand, Seijiro Mori, Koutaro Yokote, Ann-Kristin Arvidsson, Anders Eriksson, Christer Wernstedt, Ulf Hellman, Lena Claesson-Welsh, and Carl-Henrik Heldin, Ludwig Institute for Cancer Research, Biomedicum, Box 595, S-751 24 UPPSALA, Sweden.

Platelet-derived growth factor (PDGF) stimulates the tyrosine kinase activity of the PDGF receptors, leading to autophosphorylation of the receptors as well as a number of cellular proteins.

Several of the proteins phosphorylated in response to PDGF, including Ras GTPase activating protein (GAP), phosphatidylinositol (PI) 3'kinase, and phospholipase C-γ (PLC-γ), contain Src homology 2 (SH2) domains, a non-catalytic region known to bind to phosphorylated tyrosine residues in proteins.

We have localized several sites of autophosphorylation to the juxtamembrane domain as well as to the tail of the PDGF ß-receptor. Phosphorylation of tyrosine residues 1009 and 1021 in the human PDGF ß-receptor, was shown to induce the binding and phosphorylation of PLC-γ. Mutant receptors, in which Tyr-1009 and Tyr-1021 were replaced with phenylalanine residues, failed to bind PLC-γ, and PDGF failed to induce phosphorylation of PLC-γ in cells expressing the mutant receptors. However, the cells expressing the mutant receptors were still able to transduce a mitogenic signal in response to PDGF.

BZ 144 SIGNAL TRANSDUCTION BY THE αβ-HETERODIMERIC FORM OF PDGF-RECEPTORS

Eva Rupp, Agneta Siegbahn*, Carl-Henrik Heldin and Lena Claesson-Welsh, Ludwig Institute for Cancer Research, Biomedical Center, S-751 24 Uppsala, and *Department of Clinical Chemistry, University Hospital, S-751 85 Uppsala, Sweden

Platelet-derived growth factor (PDGF) is a mitogen for connective tissue cells. Three isoforms of PDGF exist, denoted PDGF-AA, -AB and -BB, which exert their biological activity by binding to two structurally related receptors with different affinities. Ligand binding induces dimerization of the receptors, either of two α-, two β-, or one α- and one β-subunit.

The properties and functions of the αβ-heterodimeric receptor complex have been compared to those of the homodimeric αα and ββ receptor complexes concerning cellular responses to PDGF, receptor autophosphorylation, substrate phosphorylation and activation. These studies have also been extended to partially inactivated αβ-receptor complexes, created through co-expression of one intact and one mutated receptor subunit. The aim of these investigations is to dissect the components involved in signal transduction by PDGF αβ-heterodimeric receptor complex.

BZ 145 SURAMIN INDUCES TYROSINE PHOSPHORYLATION AND INHIBITS CELLULAR PROLIFERATION IN A HUMAN B-LYMPHOBLAST CELL LINE, O. Sartor and C.A. McLellan. Clinical Pharmacology Branch, National Cancer Institute, Bethesda, MD 20892

Suramin, an experimental anti-cancer agent, has previously been reported to block growth factor-mediated signal transduction. More recently, suramin has been reported to induce tyrosine phosphorylation in a variety of adenocarcinoma cell lines (Sartor et al, J. Clinical Investigation, In Press). In lymphoid cell lines, the pattern of tyrosine phosphorylation is distinct from that of adenocarcinomas. One lymphoid cell line, with B-lymphoblast features (IM-9), was selected for a more detailed analysis. In this cell line, suramin rapidly (in one minute) induces tyrosine phosphorylation of several distinct proteins. Analyses indicate that GAP and its associated proteins (p62 and p190), PI 3′ kinase, and $p95^{vav}$ are among those phosphorylated. The enzymes, kinase(s) and/or phosphatase(s), mediating these alterations are as yet unidentified. In addition to inducing tyrosine phosphorylation of these putative signal transducing proteins, suramin markedly inhibits cellular proliferation. Some polysulfated compounds (pentosan polysulfate and protamine sulfate), but not all (heparin), induce a similar pattern of tyrosine phosphorylation, but do not inhibit IM-9 growth. Thus, although suramin induces both growth inhibition and tyrosine phosphorylation, it is unlikely that these events are causally linked in the IM-9 cell line.

BZ 146 TRANSMEMBRANE SIGNALLING BY AN INSULIN RECEPTOR LACKING A CYTOPLASMIC β-SUBUNIT DOMAIN, Toshiyasu Sasaoka, Yasumitsu Takata, and Jerrold M. Olefsky, Div. of Endo, Univ. of Cal. San Diego, 92093 and the VA Med. Center, La Jolla, Cal. 92161

We have previously shown that Rat1 fibroblasts expressing insulin (I) receptor (R) mutants truncated by 365 amino acids (HIRΔ978), thereby deleting over 90% of the cytoplasmic domain, displayed enhanced I sensitivity with regard to glucose incorporation into glycogen, AIB uptake, DNA synthesis, and S6 kinase activity. To explore this, we have studied the mechanisms of the enhanced signalling properties. HIRΔ978 receptors were processed normally to homodimers which were expressed at the cell surface and bound I with normal affinity. The formation of hybrid insulin: IGF-I receptors did not account for the enhanced signalling properties of I receptors in HIRΔ978 cells. As expected, the truncated receptor was inactive with respect to autophosphorylation and kinase activity toward the exogenous substrate poly Glu^4:Tyr^1. Strikingly, I stimulation of HIRΔ978 cells led to increased phosphorylation of the endogenous substrate pp185. An agonistic monoclonal antibody (Ab) specific for the human IR stimulated I action in fibroblasts expressing wild type human IRs, but had no effect on either HIRΔ978 or Rat1 parental cells. This indicates that the enhanced I signalling is mediated through the endogenous IRs. Furthermore, preincubation with this Ab led to internalization of HIRΔ978 receptors, and subsequent studies of glucose incorporation into glycogen in these Ab pretreated HIRΔ978 cells showed that the increased I sensitivity was abolished and was comparable to that of Rat1 parental cells. In summary, despite the absence of a functional cytoplasmic β-subunit domain, the truncated IRs at the cell surface confer enhanced I sensitivity by augmenting the signalling properties of the endogenous rodent IRs at the level of endogenous substrate phosphorylation.

BZ 147 ANALYSIS OF RAS-ACTIVATION BY MUTANT PDGF AND IL-2 RECEPTORS,

Takaya Satoh[1], Wendy J. Fantl[2], Jaime A. Escobedo[2], Lewis T. Williams[2], Yasuhiro Minami[3], Tadatsugu Taniguchi[3], and Yoshito Kaziro[1], [1]DNAX Research Institute, Palo Alto, CA 94304, [2]Department of Medicine, University of California San Francisco, San Francisco, CA 94143, [3]Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan

Ras protein acts as a transducer of signals controlling growth and differentiation in many types of cells including fibroblasts and lymphoid cells. The PDGF receptor binds GAP, PI3-kinase, and PLCγ, as well as induces rapid activation of Ras in a ligand-dependent manner. We examined the effect of point mutations of the receptor on the activation of Ras. PDGF-R (Y739F), which is incapable of binding and phosphorylating GAP, but can stimulate DNA synthesis, still retains the ability to activate Ras. On the other hand, PDGF-R (Y708/719F), which can neither interact with PI3-kinase nor stimulate DNA synthesis, is incapable of activating Ras. The results suggest that PI3-kinase, but not GAP, plays an important role for the transduction of signals from the PDGF receptor to Ras.

The growth signal from IL-2 is mediated by Ras in T cells. Analyses of Ras-activation in response to mutant IL-2-R β subunits have shown that two (serine-rich and acidic) domains, which are indispensable for IL-2-dependent activation of a tyrosine kinase, are important for the activation of Ras. These domains are also required for the induction of fos/jun expression, although the acidic region is not essential for DNA synthesis. Therefore, it is likely that, in IL-2 systems, tyrosine phosphorylation of certain cellular proteins leads to the activation of Ras followed by fos/jun induction, although it is not clear what kinds of regulatory molecules function downstream of a tyrosine kinase.

BZ 148 CHARACTERIZATION OF A KGF RECEPTOR INVOLVED IN THE INDUCED EPITHELIO - MESENCHYMAL TRANSITION OF RAT CARCINOMA CELLS

Pierre Savagner, Ana Maria Vallés, Jacqueline Jouanneau, and Jean Paul Thiery. Laboratoire de Physiopathologie du Développement, CNRS-ENS, 46 rue d'Ulm, 75230 Paris Cedex 05 France.

We described previously that acidic FGF (aFGF) treatment activated a rat bladder carcinoma cell line NBTII to undergo transition from epithelial to mesenchymal phenotype (EMT). Such a transition is found at different stages of development, and also during pathological events such as metastasis initiation. NBTII EMT was also stimulated by KGF, but not by basic FGF (bFGF). Interestingly, induced EMT only occured in cells grown at low density. Conversely, aFGF act specifically as a growth stimulating factor in high confluency cells, but not in low confluency cells undergoing EMT. Ligand activation was mediated through high affinity receptors that we characterized by their binding affinities. A high affinity receptor very similar to mouse KGFR was cloned from NBTII cells and from rat lung cells, showing similar sequence. Two FGF receptors, FGFR1 (Flg) and FGFR2c (Bek), already shown to bind to aFGF and bFGF were transfected into NBTII cells. Transfected cells were activated to undergo EMT by aFGF as well as by bFGF, when grown at low density. In addition, they were growth-stimulated when grown at high density. Binding studies indicated that the transfected cells could recognize bFGF as well as aFGF. Since they included intracellular domains (carboxy domains) similar to the NBTII native receptors, we concluded that the same receptor was mediating a distinct activation process in cells grown at low or high density. Cycloheximide and actinomycin D studies suggested that early protein synthesis before 2-3 hours was required in both cases to allow EMT induction. Such aFGF-induced early response factors, since they appear to be specific for EMT, could represent a new family of factors involved in extra- or intra-cellular message transduction.

BZ 149 LIGAND-INDUCED BINDING OF P85 AND PI-3 KINASE ACTIVITY TO C-KIT: ESSENTIAL ROLE OF TYROSINE 719

Serve, H., Hsu, Y.C., Huang, E., and Besmer, P., Molecular Biology Program, Sloan Kettering Institute, and Cornell University Graduate School of Medical Sciences, New York, NY 10021

The receptor tyrosine kinase c-kit is thought to mediate its diverse effects on different cell lineages by association and activation of distinct second messenger systems. The immediate events after binding of kit ligand (KL) to the receptor include the autophosphorylation of c-kit, its association with an 85 kDa subunit of the PI-3 kinase and the activation of the enzyme.

We examined the association and activation of PI-3 kinase with the c-kit protein product. In order to define the binding site of p85, the noncatalytic subunit of PI-3 kinase, we substituted each of the putative tyrosine phosphorylation sites in the kinase insert region of c-kit with phenylalanine (YF702, YF 719, YF728 and YF745, respectively) and expressed the proteins in COS-1 cells.

The results indicate, that upon stimulation of the cells with KL (1) wildtype c-kit is readily autophosphorylated. The autophosphorylation is not diminuished significantly in any of the four mutations. (2) P85 and PI-3 kinase activity associate with wildtype c-kit as well as with the mutants YF702, YF728 and YF745. Ligand-induced association of p85 and PI-3 kinase activity with c-kit are abolished in YF719. This is not due to different levels of expression of p85 or c-kit: immunoprecipitation of the cell lysates of the same experiments with anti-p85 or anti-c-kit antibodies show similar expression of these proteins in all samples. (3) C-kit-receptor bound p85 is not phosphorylated on tyrosine.

Our results indicate, that tyrosine 719 in the kinase insert within the YMDM motif plays an important role for binding of p85, and that its phosphorylation is a prerequisite for binding of p85 and subsequent activation of PI-3 kinase. The role of tyrosine 719 and PI-3 kinase activation in mediating the different receptor functions of c-kit are currently under investigation.

BZ 150 FOUR ALTERNATIVELY SPLICED ltk TYROSINE KINASES DIFFER UPSTREAM OF THEIR TRANSMEMBRANE DOMAIN AND ARE RETAINED IN THE ENDOPLASMIC RETICULUM, Allard J. Snijders, Volker H. Haase and Andre Bernards, Molecular Genetics Laboratory, Massachusetts General Hospital Cancer Center and Harvard Medical School, Building 149, 13th Street, Boston, MA 02129

Ltk is a member of the insulin group of transmembrane tyrosine kinases that is expressed in pre-B lymphocytes and in the brain. We have isolated two alternatively spliced mouse lymphocyte and brain ltk cDNAs that predict unusually small receptor kinases, which differ upstream of their transmembrane domain and which use CUG translational start codons. Two additional alternatively spliced mouse neuroblastoma ltk cDNAs, by contrast, include regular AUG start codons and predict more conventional receptors with much larger segments upstream of their transmembrane segments. The latter cDNAs include five upstream exons that are absent from the lymphoid and brain mRNAs and may represent the products of an upstream transcriptional promoter. The lymphoid form of ltk protein was recently found to reside in the endoplasmic reticulum (ER), where its kinase activity was reported to respond to changes in redox potential (Bauskin et al., Cell 66:685-696, 1991). Our data suggest that perhaps all ltk proteins share this unusual localization, suggesting that ltk proteins may have unique roles in signal transduction.

BZ 151 DEGENERATE PHOSPHOPEPTIDE LIBRARIES MAP MOTIFS FOR SH2 DOMAIN RECOGNITION, Zhou Songyang, Steven E. Shoelson, Manas Chaudhuri, Gerald Gish, Tony Pawson, Brian Schaffhausen and Lewis C. Cantley, Research Division, Joslin Diabetes Center, Harvard Medical School, Boston, MA 02215, Div. of Molecular and Developmental Biology, Mount Sinai Hospital, Toronto, Ontario, M5G 1X5, Canada, Physiology and Biochemistry Departments, Tufts University School of Medicine, Boston, MA 02111

A key event in signaling by growth factors and other agents that activate protein-tyrosine kinases is the assembly of protein complexes via association of src homology 2 (SH2) domains with specific tyrosine-phosphorylated proteins. Here we present a rapid technique for predicting the sequence specificity of individual SH2 domains. Phosphotyrosine-containing peptides degenerate at the three positions downstream of the phosphotyrosine were affinity-selected with SH2 domains on agarose beads. Sequences of the selected peptide mixture were compared to the sequence of the original mixture. The N- and C-terminal SH2 domains of the 85 kd subunit of phosphatidylinositol 3-kinase (PtdIns 3-Kinase) selected peptides with sequences phosphoTyr-(Met/Val/Ile/Glu)-X-Met and PhosphoTyr-X-X-Met respectively, consistent with known binding sites for these domains (X indicates a residue with low amino acid selectivity). In contrast, the optimal peptide for the SH2 domain of $pp60^{c\text{-}src}$ was Phosphotyr-Glu-Glu-Ile. Synthetic phosphopeptide based on the derived sequences bound tightly and specifically to the SH2 domains of PtdIns 3-kinase and pp60c-src respectively. This technique allows a rapid prediction of which SH2 domain-containing proteins will assemble with specific receptors and the domain of the receptor involved.

BZ 152 MUTATIONS OF THE HUMAN *KIT* (MAST/STEM CELL GROWTH FACTOR RECEPTOR) GENE IN HUMAN PIEBALDISM. Richard A. Spritz[1], Stuart A. Holmes[1], Seung-Taek Lee[1], Kathleen M. Strunk[1], Jennifer Lu-Kuo[2], David C. Ward[2], and Michael R. Altherr[1]. [1]Dept. of Medical Genetics, Univ. of Wisconsin, Madison, WI 53706; [2]Dept. of Genetics, Yale Univ. Sch. Medicine, New Haven, CT 06510; and [3]Dept. of Biological Chemistry, Univ. of California, Irvine, CA 92717.

Piebaldism is a rare autosomal dominant disorder of pigmentation characterized by congenital patches of white skin and hair from which melanocytes are absent. A similar disorder of mouse, "dominant white spotting" (W), results from mutations of the *Kit* gene, which encodes the receptor tyrosine kinase for the mast/stem cell growth factor. We find that human piebaldism likewise results from *KIT* gene mutations. In a patient with piebaldism plus chromosomal deletion 4q12q21.1, we demonstrated deletion of the *KIT* gene, as well as other PTK genes located nearby. We also identified point mutations of the *KIT* gene in 9 unrelated families with piebaldism. The specific phenotypes associated with these missense, frameshift, and splice site mutations suggest likely pathologic mechanisms. Furthermore, in 3 three additional unrelated patients we found identical 4-base deletions, resulting in frameshifts. This suggests that this region of the *KIT* gene is predisposed to this recurrent frameshift via a common mechanism.

We also used RT-PCR to define the repertoire of PTK mRNAs expressed by normal primary human melanocytes. By DNA sequence analysis of 608 independent PTK cDNAs, we identified 25 different PTK mRNAs expressed in human melanocytes. Five are receptors for known ligands that stimulate proliferation of melanocytes *in vitro*. Eight are entirely novel. We assayed the expression of all 25 PTK mRNAs in various human tissues by northern blot hybridization and mapped all of the corresponding PTK genes to specific human chromosomes. Surprisingly, many mapped to chromosome 4. By fluorescent in situ hybridization we mapped a cluster of PTK genes to 4q12. We have constructed a partial map of this PTK gene cluster, and find that one of these genes, *JAK1*, is interrupted by an inherited chromosomal translocation involving 4q12; this may provide a possible clue to the function of this non-receptor PTK.

BZ 153 THE TRK RECEPTOR SPECIFICITIES OF NGF, BDNF, NT-3, AND NT-4/5 IN FIBROBLASTS, PC12 CELLS, AND NEURONS, Trevor N. Stitt, Nancy Y. Ip, David J. Glass, James Fandl, Lloyd A. Greene*, and George D. Yancopoulos, Regeneron Pharmaceuticals, Inc., 777 Old Saw Mill River Road, Tarrytown, New York, 10591; * Dept. Pathology, College of Physicians and Surgeons, Columbia University, New York, NY 10032

Neurotrophic factors play important roles during the development and maintenance of the nervous system. The recent expansion of the neurotrophin family and the identification of Trk tyrosine kinases as neurotrophin receptors have, however, been accompanied by several discrepancies concerning the total number of neurotrophins and their receptor interactions. For example, independent published studies reporting the cloning of a novel neurotrophin gene from rat and human (NT-4/5) have disagreed whether this represents the mammalian homologue of the previously identified Xenopus NT-4 or a novel member of the neurotrophin family. The disagreements concerning the reported bioactivities of the potential mammalian homologue of xNT-4 also reflect similar discrepancies about the actions of the other neurotrophins.

We have exploited a battery of approaches, some of which depend on highly purified neurotrophin preparations, to qualitatively and quantitatively explore the specificity with which the neurotrophins interact with their receptors in fibroblasts, PC12 cells, as well as in nervous system tissue. Our findings reveal that rat and human NT-4/5 are indeed similar to xNT-4 in their ability to interact specifically with TrkB but that these interactions can be qualitatively distinguished from those of BDNF with TrkB, and that NGF and NT-3 are the strongly preferred ligands for TrkA and TrkC, respectively. Furthermore, we report that all of the Trks are capable of mediating neurotrophin actions at physiologically relevant doses of their preferred ligands in non-neuronal cells, arguing against a role for neuronally restricted accessory molecules (such as p75-LNGFR) in creating a high affinity receptor capable of responding to lower concentrations of the neurotrophins. However, we find that a neuronal environment does seem to restrict the Trks in their ability to respond to their non-preferred ligands.

BZ 154 CONSTRUCTION AND EXPRESSION OF AN EGFR/*eph* CHIMAERIC RECEPTOR, Nadia L. Tuzi, Helen C. Hurst and William J. Gullick, ICRF Oncology Group, Hammersmith Hospital, Du Cane Road, London W12 0NN, U.K.

A new receptor tyrosine kinase family has recently been described and consists of five members, *eph* being one. Overexpression of *eph* in NIH 3T3 cells has been shown to be transforming. No ligands have yet been identified for any of these receptors and therefore to enable ligand induced signal transduction of *eph* to be studied a chimaeric receptor consisting of the extracellular domain of the epidermal growth factor receptor and the transmembrane and intracellular domain of *eph* has been constructed. This chimaeric receptor has been expressed transiently in COS cells and stably in CHO and NIH 3T3 cells and it has been demonstrated to possess tyrosine kinase activity. These cell lines will allow studies of ligand induced mitogenesis and association of various intracellular proteins such as PLC-gamma, P13K and GAP with the stimulated chimaeric receptor. A polyclonal antibody which recognises *eph* has been raised and will assist in these studies.

BZ 155 THE ROLE OF THE INSULIN RECEPTOR IN REGULATING CELL GROWTH. Birgitte Ursø[1], Birgitte S. Wulff[1] og Steen Gammeltoft[1]. [1]Dep. of Clinical Chemistry, Bispebjerg Hospital, DK-2400 Copenhagen NV, Denmark.

Insulin and insulin-like growth factor I (IGF-I) are homologous peptides with receptors which are very similar. Insulin regulates metabolism whereas IGF-I stimulates cell division and differentiation in the organism. Both receptors comprises protein tyrosine kinases in their cytoplasmic part.

The aim of the project is to investigate the correlation between the number of insulin receptors and their ability to stimulate DNA synthesis, cell growth and cellular transformation. cDNA for the human insulin receptor were transfected into NIH 3T3 mouse fibroblast cells and stable clones with varying receptor numbers were isolated. The native cell line has about 15.000 insulin receptors.

The sensitivity to insulin is increased (from $ED_{50}=10^{-7}$ M to 10^{-9}M) and the maximal response to insulin is drastically raised with 1,000,000 receptors present, whereas the response is only slightly raised with 200,000 receptors. The insulin-stimulated growth rate of the transfected cells is increased and they show an insulin-dependent transforming ability. IGF-I is a potent stimulator of DNA synthesis through the 110.000 native IGF-I receptors.

We conclude that high levels of insulin receptors mediate a cellular growth response comparable to that of IGF-I receptors. The insulin receptor show only 2-3 fold lower potency. This indicates that it could be the signaling machinery of the differentiated cells in the organism that differs and not the signaling abilities of the two receptors.

BZ 156 **FIBROBLAST GROWTH FACTOR RECEPTOR-4 SHOWS NOVEL FEATURES IN GENOMIC STRUCTURE, LIGAND BINDING AND SIGNAL TRANSDUCTION**

Satu Vainikka, Juha Partanen, Paola Bellosta[1], Francois Coulier[2], Claudio Basilico[1], Michael Jaye[3] and Kari Alitalo[4] Cancer Biology Laboratory Dept. Pathology and Virology, University of Helsinki, 00290 Helsinki, FINLAND, [1]NYU Medical Center, New York, [2]INSERM Unite 119, Marseille, France, [3]Rhone-Poulenc Central Research, Collegeville, PA.

Fibroblast growth factor (FGF) receptor (FGFR) gene family consists of at least four receptor tyrosine kinases which transduce signals important in a variety of developmental and physiological processes related to cell growth and differentiation. Here we have characterized the binding of different FGFs to FGFR-4 (1). Our results establish a FGF binding profile for FGFR-4 with aFGF having the highest affinity, followed by K-FGF/*hst*-1 and bFGF. In addition, FGF-6 was found to bind to FGFR-4 in ligand competition experiments. Interestingly, the FGFR-4 gene was found to encode only the prototype receptor in a region where both FGFR-1 and FGFR-2 show alternative splicing leading to differences in their ligand binding specificities and to secreted forms of these receptors. Ligands binding to FGFR-4 induced receptor autophosphorylation and phosphorylation of a set of cellular polypeptides, which differed from those phosphorylated in FGFR-1 expressing cells. Specifically, the FGFR-1 expressing cells showed a considerably more extensive tyrosine phosphorylation of PLC-γ than the FGFR-4 expressing cells. Structural and functional specificity within the FGFR family exemplified by FGFR-4 may help to explain how FGFs perform their diverse functions.

1. Partanen, J., Mäkelä, T., Eerola, E., Korhonen, J., Hirvonen, H., Claesson-Welsh, L. and Alitalo, K. FGFR-4, a novel acidic fibroblast growth factor receptor with a distinct expression pattern. ***EMBO J.*** 10: 1347-1354, 1991.

BZ 157 TYROSINE 1021 AND 1009 ARE PHOSPHORYLATION SITES IN THE CARBOXYTERMINUS OF THE PDGF RECEPTOR β SUBUNIT, AND ARE REQUIRED FOR BINDING OF PHOSPHOLIPASE Cγ AND A 64 KD PROTEIN, RESPECTIVELY. Mindaugas Valius, Chantal Bazenet and Andrius Kazlauskas, National Jewish Center, Dept of Pediatrics, 1400 Jackson Street, Denver, Colorado 80206

Binding of platelet-derived growth factor (PDGF) to the PDGF receptor (PDGFR) β subunit triggers receptor tyrosine phosphorylation and the stable association of a number of signal transduction molecules, including phospholipase C γ (PLCγ), the GTPase activating protein of *ras* (GAP) and phosphatidylinositol 3 kinase (PI3K). Previous reports have identified three PDGFR tyrosine phosphorylation sites in the kinase insert domain that are important for stable association of GAP and PI3K. Here we present data for two additional tyrosine phosphorylation sites, Y1009 and Y1021, that are both in the carboxyterminal region of the PDGFR. Characterization of PDGFR mutants in which these phosphorylation sites were substituted with phenylalanine (F) indicated that Y1021 and Y1009 are required for the stable association of PLCγ and a 64 kd protein, respectively. To determine the biological consequences of failure to associate with PLCγ, we measured PDGF-dependent inositol phosphate production and initiation of DNA synthesis. The PDGFR mutants that failed to associate with PLCγ were not able to mediate the PDGF-dependent production of inositol phosphates. Surprisingly, the F1021 receptor mediated readily detectable levels of PDGF-dependent PLCγ tyrosine phosphorylation. Thus the production of inositol phosphates requires not only PLCγ tyrosine phosphorylation, but also its association with the PDGFR. Comparison of the mutant PDGFRs' ability to initiate PDGF-dependent DNA synthesis indicated that failure to associate with PLCγ and produce inositol phosphates diminished the mitogenic response by 30%. In contrast, preventing the PDGFR from binding the 64 kd protein did not compromise PDGF-triggered DNA synthesis at saturating concentrations of PDGF. We conclude that phosphorylation of the PDGFR at Y1021 is required for the stable association of PLCγ to the receptor's carboxyterminus, the production of inositol phosphates, and initiation of the maximal mitogenic response.

BZ 158 ANALYSIS OF AUTOPHOSPHORYLATION SITE MUTANTS IN THE MURINE CSF-1 RECEPTOR EXPRESSED IN RAT FIBROBLASTS. Peter van der Geer and Tony Hunter. Molecular Biology and Virology Laboratory, The Salk Institute. P.O. Box 85800, San Diego CA 92186-5800.

The receptor for CSF-1 is a ligand-activated protein-tyrosine kinase that is involved in regulation of proliferation, differentiation and survival of macrophages and their direct precursors.

Stimulation of the receptor with CSF-1 leads to rapid autophosphorylation at several tyrosine residues. It is currently thought that autophosphorylation at certain sites facilitates the specific interaction of growth factor receptors with SH-2 domain containing substrate molecules. We and others have identified several sites of autophosphorylation in the CSF-1 receptor. Three of those sites, Tyr 697, Tyr 706 and Tyr 721, are present within the CSF-1 receptor kinase insert domain. This domain is important for CSF-1 dependent transformation of Rat-2 fibroblasts. Tyr 721 has been identified as the binding site for the p85 subunit of PI 3'-kinase. Tyr 807, which is present in the second half of the kinase domain and is conserved in all protein-tyrosine kinases, is also autophosphorylated in response to CSF-1. We have generated a number of mutant receptors in which individual phosphorylation sites have been changed. Mutants lacking individual or combinations of phosphorylation sites were expressed in Rat-2 fibroblasts and are currently being tested for their ability to induce transcription of early response genes and changes in growth rate and cell morphology in response to CSF-1.

BZ 159 ISOLATION OF A CHICKEN cDNA ENCODING A NOVEL RECEPTOR PROTEIN TYROSINE KINASE, Michael F. Verderame and Doyle Siever, Department of Microbiology and Immunology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033

Protein tyrosine kinases (PTK's) play a crucial role in the regulation of cell growth and development. Mutations resulting in aberrant expression or altered regulation of PTK's may lead to oncogenesis. In order to identify novel PTKs, a phage λgt11 chicken embryo cDNA expression library was screened with anti-phosphotyrosine antibodies. A primary screening of approximately 500,000 phage yielded 170 positive clones. At least seven subsets of tyrosine kinases have been identified among the pool of clones. Limited nucleic acid sequence determination has revealed at least one novel receptor-type PTK.

Nucleotide sequence analysis demonstrates that this novel gene is most likely a new member of the Eph/Elk sub-family of receptor PTKs. Members of this subfamily are expressed in a wide variety of tissues including brain, liver, lung, kidney, and testis as well as human epithelial tumors and pre-B and T-cell lines. Additionally, developmentally regulated expression has been observed in chicken embryos (notably in brain and muscle) for at least one member of this sub-family.

Efforts are currently being directed towards obtaining and sequencing a full-length cDNA clone encoding this novel kinase, Northern blot analysis to identify developmental and tissue specific regulation of the mRNA, and generation of antibodies against the protein product encoded by this gene. Efforts to clone the murine and human homologue of this cDNA are also underway.

BZ 160 DISTINCT PROTEIN TYROSINE PHOSPHORYLATION DURING MITOGENESIS IN SV40-TRANSFORMED CELLS INDUCED BY INSULIN OR VANADATE, Hanlin Wang and Robert E. Scott, Department of Pathology, The University of Tennessee Health Science Center, Memphis, TN 38163

Insulin and vanadate induce mitogenesis in SV40 large T antigen-transformed CSV3-1 cells by distinct signal transduction mechanisms that involve protein tyrosine kinase activity. To further study these processes, changes in protein tyrosine phosphorylation induced by insulin and vanadate were investigated. Using combined immunoprecipitation and Western blotting techniques with anti-phosphotyrosine antibodies, we report that although the insulin receptor beta-subunit is phosphorylated within 2 min after insulin stimulation in both quiescent transformed CSV3-1 and non-transformed 3T3 T cells, only CSV3-1 cells show enhanced phosphorylation of a 51 kDa and a 61 kDa protein within 5 min. In vanadate-stimulated CSV3-1 cells, a pattern of phosphorylation was observed that was also distinct from 3T3 T cells. Vanadate does not initially increase phosphorylation, however, after 30 min increased phosphorylation of a pre-existing 72 kDa protein is observed. In addition, the phosphorylation of a 55 and a 64 kDa protein is dramatically enhanced after 60 min. Furthermore, treatment of CSV3-1 cells with genistein, a tyrosine kinase inhibitor, abolishes the effects of vanadate but not the effects of insulin. These observations are consistent with our previous findings that mitogen-induced c-jun expression can be blocked by genestein in vanadate-stimulated CSV3-1 cells but not in insulin-stimulated cells. These data together indicate that the mitogenic effects of insulin and vanadate that are specific for SV40-transformed CSV3-1 cells are mediated by different tyrosine phosphorylation-dependent mechanisms. They also suggest that protein tyrosine phosphorylation is differentially regulated in SV40-transformed 3T3 T cells and non-transformed 3T3 T cells.

BZ 161 ROLE OF EGF RECEPTOR AUTOPHOSPHORYLATION SITES IN SIGNALING MITOGENESIS, Alan Wells. Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294.

The epidermal growth factor receptor (EGFR) possesses an intrinsic tyrosine kinase activity and at least five potential autophosphorylation sites. Upon stimulation by ligand, the kinase is triggered and the receptor is noted to associate with *src* homologous SH2 domains present in downstream effector molecules. However, the role of specific interactions in signaling mitogenesis is uncertain at present. It has been shown that an EGFR mutant from which all autophosphorylation sites had been removed induced mitogenesis, suggesting that the interactions per se are not necessary for mitogenesis. It is still possible that mitogenic signaling may be augmented via messengers recruited by phosphotyrosyl-SH2 interactions.

EGFR constructs were generated in which the carboxyl-termini containing all the potential autophosphorylation sites were removed and replaced individually by nine amino acid-long sequences containing four of the autophosphorylation sites. In parallel constructs, the targeted tyrosines were encoded as phenylalanines. These EGFR were expressed stably in NR6 fibroblasts which lack endogenous EGF receptors. The selected cells were examined for EGF-stimulated cell growth, kinase activity, and autophosphorylation.

In all cases, EGFR with kinase activity stimulated cell growth. This was independent of receptor tyrosyl-autophosphorylation. Studies are underway to determine if the phosphotyrosyl-containing EGFR result in either a greater or a more sensitive growth response. Further, the ability of these mutant EGFR to physically associate *in vivo* with certain SH2-domain containing proteins will be presented.

In conclusion, these results support the findings that autophosphorylation-mediated interactions between the EGFR and downstream signaling molecules are not necessary for ligand-induced mitogenesis. Thus, these latter pathways may be associated with triggering other cellular responses.

BZ 162 ASSOCIATION OF SUBSTRATE PROTEINS WITH THE INSULIN RECEPTOR. Zhang Wengang, Jeffrey D. Johnson, William J. Rutter. Hormone Research Institute, UC. San Francisco, Ca 94143-0534

Insulin binding to the extracellular portion of the insulin receptor leads to activation of the intracellular kinase and tyrosine phosphorylation of a number of cellular proteins. It has been difficult, however, to describe which phosphorylation events are the result of direct interaction with the insulin receptor kinase and therefore likely to represent physiologically relevant signal transducing molecules. Since it appeared that some targets of the insulin receptor kinase may interact transiently with the activated receptor, we have used a novel method for rapidly purifying an epitope modified insulin receptor under mild conditions to detect a set of insulin receptor associated substrate (IRAS) proteins with molecular weights of 240, 190, 120, 85, 60, 45, and 42. These proteins interact specifically with the activated form of the receptor and remain associated with the receptor in the presence of EDTA. A subset of the IRAS proteins associate with the insulin receptor to a greater extent when obtained from cells which have not been exposed to insulin, perhaps indicating that they are modified in some way in insulin treated cells. Upon restoration of ATP/Mg^{++}, the IRAS proteins are tyrosine phosphorylated within the complex *in vitro* and dissociate from the activated receptor complex. We also found p120 GAP and the p85 subunit of PI3K associated with the activated FIR. The p85 protein, like the IRAS proteins, is stably bound in the presence of EDTA and released from the complex in the presence of ATP/Mg^{++}. This behavior may explain the transient association of PI3K activity with the activated insulin receptor. These studies suggest that insulin signal transduction proceeds through transient interactions with substrate molecules whose phosphorylation may lead to dissociation from the complex.

Non-Kinase Receptors

BZ 200 A DOMINANT NEGATIVE ERYTHROPOIETIN RECEPTOR (EPO-R) POLYPEPTIDE ABROGATES EPO-DEPENDENT GROWTH OF A HEMATOPOIETIC CELL LINE, Dwayne Barber, John DeMartino, Mark Showers, and Alan D'Andrea, Division of Pediatric Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

The erythropoietin receptor (EPO-R), a member of the cytokine receptor superfamily, can be activated to signal cell growth by binding either EPO or gp55, the Friend Spleen Focus-Forming Virus (SFFV) glycoprotein. Although the EPO-R cytoplasmic tail does not contain a tyrosine kinase catalytic domain, EPO-binding or gp55-binding results in the rapid induction of tyrosine kinase activity. We have utilized a murine interleukin-3 (IL-3) dependent cell line, Ba/F3, transfected with the EPO-R cDNA, to analyze EPO-dependent tyrosine phosphorylation. Ba/F3-EPO-R cells, stimulated with EPO or gp55, result in tyrosine phosphorylation of the EPO-R itself (p72) and of p97 and p38 substrates. Ba/F3-EPO-R(T), which express a truncated, cytoplasmic tail-less form of EPO-R (EPO-R(T)), bind EPO but do not grow in EPO or display EPO-induced tyrosine kinase activity. Overexpression of the EPO-R(T) polypeptide in Ba/F3-EPO-R cells abolishes EPO-dependent growth and EPO-R tyrosine phosphorylation without affecting IL-3 dependent growth or IL-3-dependent tyrosine kinase activity. Moreover, the EPO-R(T) forms stable complexes with the full length EPO-R polypeptide. In conclusion, the EPO-R(T) is a dominant negative protein which abolishes wild-type EPO-R signalling and EPO-R tyrosine phosphorylation, perhaps by forming non-productive heterodimeric complexes with the full length EPO-R.

BZ 201 CNTF INDUCES PHOSPHORYLATION OF MULTIPLE PROTEINS AND ACTIVATES PATHWAYS IN COMMON WITH OTHER GROWTH FACTORS AND NEUROTROPHINS, Teri G. Boulton, Neil Stahl, George D. Yancopoulos. Regeneron Pharmaceuticals Inc. Tarrytown, New York 10591-6707

Ciliary neurotrophic factor (CNTF), originally identified for its ability to support the survival of some species of neurons, is structurally unrelated to the NGF family of neurotrophins. Instead, CNTF is more homologous to the cytokines leukemia inhibitory factor (LIF), interleukin-6 (IL-6), and oncostatin M (OSM). The receptor for CNTF is composed of three components, two of which make up the functional LIF receptor. The signal transducing components for the LIF receptor are gp130 and LIFRß. In addition to gp130 and LIFRß, CNTF requires its specific binding protein, CNTFRα, to transduce the signal to the cytoplasm. Thus, LIF and CNTF are capable of activating the same pathways. None of these receptor proteins contains an intrinsic tyrosine kinase domain, but both CNTF and LIF induce rapid phosphorylation of proteins on tyrosine residues.

Neuronally-derived cell lines were used to examine the patterns of phosphorylation and the signal transduction pathways activated by CNTF and LIF. LIFRß and gp130 both become phosphorylated rapidly upon treatment of cells with factor. The other major phosphoproteins are pp150, pp122, pp82, pp72 and pp42 and the time course of phosphorylation of these proteins with the exception of pp42 is visible 0.5 minutes after treatment of cells with CNTF. pp82 appears as a single tyrosine phosphoprotein initially, becomes a doublet with pp83 by 5 minutes then is only the 83 kDa species by 10 minutes. The 42 kDa protein has been identified as ERK2 and its time course of phosphorylation and activation coincides with the conversion of the 82 kDa protein to the slower migrating form. Phosphorylation of ERK1 and ERK2 in response to CNTF compared to EGF appears to be slower and the phosphorylation is sustained longer. Phosphorylation of phospholipase C gamma 1 and the GTPase activating protein, ras GAP, in response to stimulation of cells with CNTF has also been examined.

BZ 202 MOLECULAR AND FUNCTIONAL ASSOCIATION OF CD2 CELL SURFACE RECEPTOR WITH ζ-CHAIN AND p59^{fyn} PROTEIN TYROSINE KINASE IN T CELLS

Paul Burn, Kurt E. Amrein, B.Schraven and Martin Gassmann, Pharmaceutical Research-New Technologies, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland

CD2 is a cell surface receptor molecule of 50 kD that is expressed on T lymphocytes, thymocytes, and natural killer cells. Numerous experiments have revealed a dual function for CD2 as a signal and adhesion molecule.The aim of the present study is to elucidate the molecules involved in the CD2 signal transduction cascade.

Using human T lymphocytes and anti-CD2 antibodies we demonstrate that triggering of CD2 results in tyrosine phosphorylation of a distinct set of cellular proteins within seconds of stimulation, suggesting the involvement of at least one protein tyrosine kinase early in CD2-mediated signal transduction events. We next show that protein tyrosine kinase activity can be co-immunoprecipitated with CD2 from detergent-extracts of T cells, and that such CD2 immune complexes contain the ζ-chain and the *src family* protein tyrosine kinases p59^{fyn} and p56^{lck}. To further explore the significance of these findings *in vivo* under physiologically relevant conditions we have performed co-capping experiments in functional human T lymphocytes using double indirect immunofluorescence microscopy. These experiments revealed a specific co-distribution of a significant fraction of the ζ-chain and p59^{fyn}, but not p56^{lck}, with CD2 cell surface receptor caps.

These results provide the first evidence for a multimolecular signalling complex which involves at least CD2 cell surface receptor, ζ-chain and p59^{fyn} protein tyrosine kinase. Furthermore they suggest that CD2-mediated signalling proceeds via a signal transduction cascade which involves ζ-chain and p59^{fyn} protein tyrosine kinase activation.

BZ 203 THE HUMAN GRANULOCYTE - MACROPHAGE COLONY - STIMULATING-FACTOR RECEPTOR IS CAPABLE OF INITIATING SIGNAL TRANSDUCTION IN NIH 3T3 CELLS. M. Eder, M. Hallek, J.D. Griffin, and T.J. Ernst. Division of Tumor Immunology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA 02115 and Medizinische Klinik, Klinikum Innenstadt, University of Muinch, FRG.

The ability of hematopoietic cytokine receptors such as granulocyte-macrophage colony stimulating factor (GM-CSF) receptor to function in non-hematopoietic cells is unknown. A series of NIH 3T3 cell lines which stably express human GM-CSF receptor (GM-CSFR) have been generated by transfection of cDNAs encoding the α and β receptor subunits. The reconstituted receptors bind human GM-CSF with high affinity (Kd=20-80 pM) and can activate some of the same signal transduction pathways known to be activated by the GM-CSFR in hematopoietic cells, including tyrosine kinase activity and transient induction of c-fos, c-myc and c-jun mRNAs. The activation of protein tyrosine phosphorylation by GM-CSF was rapid (< 1 min) and transient (peaking at 5-20 min), and resulted in phosphorylation of proteins of 42, 44, 52/53, 60, 90, 120, and 140-150 kD. Some of these proteins comigrated with proteins from hematopoietic cells which were phosphorylated on tyrosine residues in response to GM-CSF. In particular, p42 and p44 were identified as mitogen activated protein kinases (MAP kinases) in both cell types. However, several of the proteins which are characteristically tyrosine phosphorylated in hematopoietic cells, such as p93 and p70, were not observed in NIH 3T3 cells. Finally, despite evidence for activation of many mitogenic signal transduction molecules, GM-CSF did not induce expression of the G1 cyclins D2 or D3, or significant proliferation of transfected NIH 3T3 cells. We conclude that in spite of evidence of signal transducing molecules which can effectively interact with the human GM-CSFR in NIH 3T3 cells, the level of activation is either below a threshold of necessary activity or at least one mitogenic signal which is essential for proliferation is missing.

BZ 204 ASSOCIATION OF TYROSINE PROTEIN KINASE ACTIVITY WITH THE CD14 ANTIGEN IN HL-60 CELLS.
Julie A. Ely, Karen G. Kabat-Stegman, Donald E. Wegemer and William S. Kloetzer. The R.W. Johnson Pharmaceutical Research Institute, San Diego, CA, 92121.
The CD14 antigen (Ag) is a glycosyl-phosphatidylinositol (G-PI) linked protein expressed primarily on leukocytes of the monocytic and, to a much lesser extent, the myeloid lineage. The CD14 Ag is reported to be a receptor for lipopolysaccharide (LPS) in association with the serum LPS Binding Protein LBP (Science 249:1429, 1990). Several reports have shown that monoclonal antibodies (MAbs) against CD14 Ag can inhibit monocyte binding of LPS/LBP complex and subsquent activation of inflammatory cytokine release. The CD14 Ag influence on the various intracellular signaling events activated by LPS are presently under investigation using HL-60 cells chemically differentiated with *calcitriol* (an inducer of the monocyte lineage) and DMSO (an inducer of the myeloid lineage). One way in which LPS might exert its effect is through a CD14 Ag-associated tyrosine protein kinase (TyrPK), as previously described for CD14 and several other G-PI linked proteins (Science 254:1016, 1991). To identify the CD14 Ag-associated TyrPK activity, cell lysates were prepared from differentiated or uninduced HL-60 cells using a digitonin-containing lysis buffer which favors non-covalent protein associations. MAbs against CD14 or unrelated Ags were used for immunoprecipitation; each sample was then *in vitro* phosphorylated by the addition of [γ-^{32}P]ATP. The labeled samples were then re-immunoprecipitated with various antibodies (against unrelated Ags, CD14 Ag or pTyr). The [^{32}P]Ags were resolved by SDS-PAGE and then visualized by autoradiography. The results show that antibody against pTyr immunoprecipitates three major *in vitro* labeled proteins (mol wts 58 Kd, 63 Kd and 83 Kd) which co-purify with CD14 Ag. LPS activation of cells prior to immunoprecipitation slightly enhances the *in vitro* phosphorylation of these proteins. Overall, these results suggest that CD14 Ag may have an associated TyrPK activity which is stimulated by LPS treatment. Phosphoamino acid analysis, identities of the [^{32}P]Ags and the mechanism of apparent LPS stimulated TyrPK activity are presently under investigation.

BZ 205 **PHOSPHORYLATION REACTIONS REGULATE ACTIVITY OF THE CONTACTINHIBIN-BINDING PROTEIN**, G. Gradl, D. Hinz, F. Oesch and R.J. Wieser, Institute of Toxicology, University of Mainz, D-65 Mainz, Germany
The growth of normal human diploid fibroblasts is regulated by a cell density-dependent mechanism. "Contact inhibition" is mediated by the integral cell membrane glycoprotein Contactinhibin[1]. A Contactinhibin-binding protein (CBP) was found by a novel aggregation assay using Contactinhibin and putative receptor fractions covalently attached to beads. This semiquantitative assay provided a useful tool for detecting this putative receptor for Contactinhibin after purification by preparative 1D and 2D SDS-PAGE and renaturation. CBP is a sialylated and phosphorylated 95 kD glycoprotein. A terminal ß-galactose on the N-glycans of Contactinhibin is required for CBP binding as well as for exerting biological activity. Transformed cells failed to respond to the Contactinhibin contact with a decrease of proliferation, indicating a defect in receptor-mediated signal transduction since those cells synthesized Contactinhibin in biologically active form.
First studies showed reduced ligand-binding of CBP derived from virus-transformed fibroblasts. A system of reversible phenotypical transformation of cultured fibroblasts by a combination of growth factors supplied data for CBP phosphorylation changes during transformation. We have evidence that cell transformation could lead to enhanced CBP phosphorylation and the loss of an up-regulation mechanism of CBP which might be important for contact inhibition.

(1) Wieser, R.J.; Schütz, S.; Tschank, G.; Dienes, H.-P.; Thomas, H. and Oesch,F.(1990) J. Cell Biol. 111, 2681-2692.

BZ 206 TNF-MEDIATED SIGNALING FOR GENE INDUCTION INVOLVES TYROSINE PROTEIN KINASE ACTIVITY
Guy Haegeman, Elke Boone and Walter Fiers, Laboratory of Molecular Biology, Gent University, Belgium

TNF is a pluripotent cytokine, which displays a cell-killing effect towards many transformed cell types. Besides this cytotoxic activity, TNF also induces the transcription of particular genes, such as the IL6 gene in the mouse fibrosarcoma cell line L929. Although activation of this IL6 gene runs parallel to the cellular toxicity exerted by TNF, the actual signaling pathway(s) for both phenomena is (are) unknown. Therefore, we have studied the effect of several tyrosine protein kinase inhibitors, acting along different mechanisms, and found that they strongly reduced the TNF-mediated induction of the IL6 gene. In contrast, the cellular toxicity exerted by TNF was not repressed by those drugs, nor was the TNF-mediated activation of the transcription factor NFκB. Previously, we already demonstrated that NFκB is not sufficient for fine regulation of TNF-induced IL6 gene expression and suggested that (an) additional factor(s) might be necessary. Taken together, our present data illustrate that tyrosine protein kinase activity is most probably required for activation of cooperative factors/signals, acting in synergy with NFκB. In conclusion, we propose that TNF-mediated signaling leading to gene induction involves at least a dual pathway. One signal consists of rapid and independent activation of NFκB; another signal, which apparently requires tyrosine protein kinase activity, leads to the activation of cooperative factors for gene transcription and can be distinguished from the cellular mechanisms resulting in TNF cytotoxicity.

BZ 207 HIGH LEVEL EXPRESSION OF GROWTH HORMONE RECEPTOR INTRACEULLAR DOMAIN IN E.COLI,
Lei Jin, Gery Baumann, Tim Clackson and James A Wells. Department of Protein Engineering, Genentech Inc. and Department of Pharmaceutical Chemistry, University of California, San Francisco, CA94143

Receptor dimerization is believed to be critical to the signal transduction of hGH. However, it is not clear at present time what kind of molecules are associating with the growth hormone receptor and responsible for tranducing the signal of growth hormone stimulation. To solve this big mystery, we have successfully expressed growth hormone receptor intracellular domain(GHR-ICD) in E.coli in a soluble form and purified it to near homogeneity. Limited proteolysis digestion experiment suggests that the GHR-ICD probably folds as a two domain protein, with the N-terminal domain more resistant to proteolysis. Indeed, the N-terminal domain of the GHR-ICD can be expressed in the same way as the full-length GHR-ICD. Considering the fact that the N-terminal domain of GHR-ICD is sufficient for transducing growth signals in FDC cells transfected with growth hormone receptor, our data suggest that the E.coli expressed GHR-ICD probably is correctly folded. By affinity purification and screening lgt11 expression libraries using the purified ICD, we are currently on the process of identifing the cellular components that are associated with the growth hormone receptor intracellular domain.

BZ 208 β_2-INTEGRIN SIGNALING IN T-CELLS THROUGH PLCγ1 IS TCR-DEPENDENT, Steven B. Kanner, Laura S. Grosmaire, Jeffrey A. Ledbetter and Nitin K. Damle, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA 98121

Stimulation of the T-cell antigen receptor complex (TCR/CD3) induces an early and transient cascade of tyrosine kinase activity. Tyrosine phosphorylation of phospholipase-Cγ1 (PLCγ1) after TCR/CD3 ligation results in its enzymatic activation, measured functionally by calcium mobilization due to the generation of inositol-trisphosphates. Here we investigated the signaling potential of the T-lymphocyte β_2-integrin (CD18, LFA-1). Cross-linking of CD18 induced both tyrosine phosphorylation of PLCγ1 and fluxing of intracellular calcium in human T-cells. Co-ligation of CD18 and TCR with monoclonal antibodies 60.3 and WT31 increased the level of PLCγ1 activation, and was accompanied by an augmentation of the amplitude of intracellular calcium mobilization with accelerated kinetics. In addition, these effects were observed in both resting and PHA-activated human T-cells. Modulation of the TCR prior to CD18 ligation showed that β_2-integrin signaling was TCR-dependent. Further, direct signaling through CD18 was tyrosine kinase-dependent as shown by inhibition with the ansamycin antibiotic herbimycin A. These results demonstrate that the T-cell integrin LFA-1 signals through common components of the transduction pathway that are linked to the TCR. The respective contributions of the α-chain (CD11a) and β-chain (CD18) of the LFA-1 integrin during T-cell signal transduction will be presented.

BZ 209 4-1BB, A NOVEL T-CELL SURFACE RECEPTOR IS ASSOCIATED WITH P56^{lck}. Young-June Kim[1,2], Karen E. Pollok[1], Zhen Zhou[1], José Hurtado[1], Jin K. Lee[1], Pasula Rajamouli[1] and Byoung S. Kwon[1,2]. [1]Department of Microbiology and Immunology and the [2]Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202.

The murine 4-1BB was isolated previously by a modified differential screening procedure, which employed both positive and negative screening to select for cDNA specific to T-lymphocytes. Structural analysis of the deduced amino acid sequence suggested that 4-1BB may be a cell surface receptor. This sequence possesses a signal peptide, a cysteine-rich extracellular domain, a transmembrane region, and a cytoplasmic tail. The cytoplasmic tail of 4-1BB was not homologous to any known kinases, but does contain the sequence, Cys-Arg-Cys-Pro which is similar to the sequence Cys-Lys-Cys-Pro that binds to the p56^{lck} tyrosine kinase in the CD4 and CD8 molecules. Recently a monoclonal antibody, 53A2, has been raised against the 4-1BB antigen and has been used to confirm by FACS analysis that 4-1BB is expressed on the cell surface of activated CD4$^+$ and CD8$^+$ T cells. Immunoprecipitation analysis with 53A2 has shown that 4-1BB is expressed as a 30 kD protein under reducing conditions. The native 4-1BB protein may exist as a dimer and even a tetramer on the cell surface, for 4-1BB is immunoprecipitated as a 55 kD dimer and a 110 kD tetramer under nonreducing conditions. The physical association between 4-1BB and p56^{lck} was examined by sequential immunoprecipitation with 53A2 and anti-p56^{lck} antibody. p56^{lck} was coimmunoprecipitated with 4-1BB from both CD4$^+$ (F1 clone) and CD8$^+$ (CTLL-2 cell line) T-cells.

BZ 210 **MULTIPLE COMPONENTS OF THE T CELL ANTIGEN RECEPTOR COMPLEX BECOME TYROSINE PHOSPHORYLATED UPON STIMULATION**
Dapeng Qian*, Irene Griswold-Prenner#, Marsha Rich Rosner#, and Frank W. Fitch*, The Committee on Immunology*, The Departments of Pathology*, Pharmacological and Physiological Sciences#, and The Ben May Institute*#, The University of Chicago, Chicago, IL 60637

Triggering of the multicomponent T cell antigen receptor (TCR) complex results in several biochemical processes which are critical for the functional activation of T lymphocytes. One common process is the tyrosine phosphorylation of several proteins, including the TCRζ chain. Using ^{32}P-biosynthetic labeling in conjunction with phosphoamino acid analysis and anti-phosphotyrosine immunoblotting, we demonstrate that in addition to TCRζ, other subunits (CD3γ, CD3δ, and CD3ε) of the TCR complex can also be tyrosine phosphorylated in response to antigen receptor stimulation. This rapid phosphorylation (which occurs as soon as 1 min after receptor engagement) was detected in several mature murine T cell subsets, including CD4+ type 1 and 2 helper cells. Therefore, tyrosine phosphorylation of multiple TCR components may be an important event during the initiation of the signalling cascade leading to T cell activation.

BZ 211 COUPLING OF GTP-BINDING TO THE T CELL RECEPTOR ζ CHAIN WITH TCR-MEDIATED SIGNAL TRANSDUCTION. Jaime Sancho*, Marcus E. Peter*, Rafael Franco*, Silvia Danielian+, Remi Fagard+, Jamilé Woods§, John C. Reed§, Malek Kamoun§ and Cox Terhorst*. *Division of Immunology, Beth Israel Hospital, and Department of Pathology, Harvard Medical School, Boston, MA 02215. +INSERM U332, Institut Cochin de Génétique Moléculaire, Paris, France. §Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA 19104-6082.

The ζ subunit of the T cell antigen receptor (TCR) binds GTP and is a well characterized substrate for a TCR activated tyrosine kinase. To examine the possible coupling of GTP-binding to ζ with TCR-mediated signal transduction, a mutant (termed J32-3.2) of the T cell line Jurkat (J32) was used. Anti-TCR/CD3 stimulation of the TCR/CD3+ J32-3.2 cells resulted in a weak stimulation of both the phosphatidyl inositol (PI) and tyrosine kinase signal transduction pathways, as measured by changes in the level of free intracellular calcium, tyrosine phosphorylation of TCR ζ, CD3-ε and ZAP-70, p56^{lck} or p59^{fyn} tyrosine kinase activity and IL-2 gene activation. The impaired responsiveness of J32-3.2 cells to anti-TCR/CD3 monoclonal antibodies correlated with a low basal level of GTP-binding to ζ. Furthermore, in J32-3.2 cells TCR activation by antibody ligation caused a weaker increase in GTP-binding to the ζ chain, as compared with that of wild-type J32 cells, which indicates for the first time that GTP-binding to ζ can be modulated by extracellular signals and suggest that the role of GTP-binding to ζ is to couple the TCR to intracellular signal transduction mechanisms.

BZ 212 DELETION ANALYSIS OF THE COMMON β SUBUNIT OF THE GM-CSF, IL-3, AND IL-5 RECEPTORS.

Noriko Sato, Kazuhiro Sakamaki, Ikuko Miyajima, Toshio Kitamura, Kenichi Arai and Atsushi Miyajima. DNAX Research Institute, Palo Alto, CA94304

GM-CSF, IL-3, and IL-5 stimulate proliferation and differentiation of hematopoietic cells through the high affinity receptors. The high affinity receptors for these cytokines are composed of a cytokine specific α subunit and the common β subunit which is shared by the three receptors. Signal transduction pathways of these cytokines include activation of tyrosine kinase, PI-3 kinase, p21ras, c-raf, and MAP kinase. These cytokines are also known to activate various nuclear protooncogenes such as c-fos, c-jun, and c-myc. Although neither the α nor β subunit has any motif of known signalling proteins, the β subunit is an essential component for signal transduction. We have generated a series of the cytoplasmic deletion mutants of the β subunit and examined their function by coexpressing with the α subunit of the GM-CSF receptor in a mouse IL-3 dependent BaF3 cell line. Whereas cells expressing the full length β subunit exhibited several prominent tyrosine phosphorylated proteins in response to GM-CSF, no significant tyrosine phosphorylation was induced by the β_{517} mutant that lacks the C-terminal 364 amino acid residues. However, the β_{517} transfectant still proliferated in the presence of GM-CSF, indicating that the major tyrosine phosphorylation is not required for proliferation. We are currently analyzing the other signals induced by these β mutants to locate the cytoplasmic domains of the β subunit responsible for those signals.

BZ 213 IL-2 RECEPTOR β CHAIN SERINE-RICH REGION IS REQUIRED FOR ACTIVATION OF PI 3-KINASE

Takuya Kanazawa, Marilyn L. Keeler and Lyuba Varticovski, Department of Biomedical Research, St. Elizabeth's Hospital, Boston, MA 02135

The intracellular domain of the IL-2 Receptor β chain contains three distinct regions. Beginning from the carboxy terminus, they have been designated as proline-rich, acidic and serine-rich regions. The receptor does not possess intrinsic protein-tyrosine kinase activity, nor contains SH2 domain recognition sequences. IL-2 binding induces activation of intracellular protein-tyrosine kinase *lck*, resulting in phosphorylation of several intracellular substrates including the β chain of the receptor. The interaction with *lck* oncoprotein was mapped to the acidic region of the IL-2R β chain and requires the presence of the kinase region of *lck*. IL-2 binding also induces association of the receptor with phosphatidylinositol 3-kinase (PI 3-kinase). We analyzed IL-3 dependent cell lines which express IL-2R wild type β chain and deletion mutants for activation of PI 3-kinase. IL-2 mediated an increase in immunoprecipitable PI 3-kinase activity from cells expressing the wild type *β* chain and from mitogenically-responsive mutants. Cells that expressed the *β* chain lacking the acidic but not the serine-rich region showed an increase in PI 3-kinase products *in vivo* in response to IL-2. These results suggest that the serine-rich region is required for interaction of IL-2R with PI 3-kinase and this interaction occurs by a mechanism distinct from association with the *lck* protein.

BZ 214 INTERLEUKIN-2 (IL-2) INDUCED TYROSINE KINASE ACTIVITY AND GENE INDUCTION BY WILD-TYPE AND MUTANT IL-2 RECEPTORS. P. Williamson, I. Merida and G. Gaulton. The University of Pennsylvania, Philadelphia, PA 19104.

High affinity IL-2 receptors (IL-2R) are composed of at least two distinct proteins, IL-2Rα (p55) and IL-2Rβ (p75). All of the known cytoplasmic signalling properties of IL-2-R are linked to the expression of the IL-2-Rβ subunit, but the intracellular sequence of events by which IL-2Rβ subunits couple to cytosolic and ultimately nuclear elements remains largely unknown. In this study we have examined the molecules associated with IL-2Rβ signalling, and subsequent effects on *c-myc* expression in CTLL-2 cells, and a sub-line of an IL-3 dependent cell line (Baf-03) which had been previously transfected with wild-type or mutated IL-2Rβ chains. The mutated β-chain was produced by deletion of the sequence which encodes amino acids 265-323, and includes the serine-rich (S) region of the cytoplamic domain. The results indicate that binding of the IL-2R by IL-2 leads to phosphorylation of the p85 sub-unit of phosphatidylinositol 3-kinase on tyrosine residues, and that the S-region of IL-2Rβ chain is essential for IL-2 induced tyrosine kinase activity and for induction of *c-myc* gene expression.

BZ 215 COMPARISON OF SIGNAL TRANSDUCTION MECHANISMS MEDIATED BY INTERLEUKIN-6 OR INTERLEUKIN-11 IN VARIOUS CELL TYPES. Yu-Chung Yang and Tinggui Yin, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN 46202.

Interleukin (IL)-11 is a bone marrow stromal cell derived cytokine with many overlapping biological activities with IL-6. Initial biochemical analysis showed that the two cytokines interact with distinct ligand binding molecules[1]. We have compared the signal transduction pathways mediated by IL-6 or IL-11 in several IL-6/IL-11 responsive cell lines (mouse preadipocyte 3T3-L1, mouse B cell hybridoma B9-TY1, rat hepatoma H-35 and human erythroleukemia TF-1) by examining protein tyrosine phosphorylation, activation of primary response genes and the role of IL-6 signal transducer, gp130, following stimulation with IL-6 or IL-11. Since tyrosine kinases and H7-sensitive kinases have been implicated to play important roles in IL-6-mediated signal transduction[2], we also examined the effects of various kinase inhibitors and activators on IL-11 mediated protein tyrosine phosphorylation and primary response gene expression. In TF-1 cells, cell proliferation, protein tyrosine phosphorylation and primary response gene expression elicited by IL-6 or IL-11 can be blocked by anti-gp130, demonstrating that IL-6 and IL-11 utilize gp130 as the common signal transducer. Comparison of protein tyrosine phosphorylation mediated by IL-6 or IL-11 has shown similar and different patterns depending on cell types examined. Despite the differences in protein tyrosine phosphorylation between IL-6 and IL-11 in certain cell types, the primary response genes activated in the same cell line are indistinguishable between IL-6 and IL-11. These results suggest that there are convergent and divergent points along the signal transduction pathways mediated by IL-6 or IL-11 and may explain some of the overlapping and unique biological functions possessed by these cytokines.

[1] Yin et al., J. Biol. Chem. 267:8347, 1992
[2] Nakajima and Wall, Mol. Cell. Biol. 11:1409, 1991

BZ 216 EARLY SIGNALLING EVENTS STIMULATED BY INTERLEUKIN-7 IN HUMAN T CELLS, Michele T. Yip-Schneider and Hal E. Broxmeyer, Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202

Interleukin-7 (IL-7) was initially identified as a pre-B cell growth factor produced by bone marrow stromal cells. Subsequently, IL-7 has been shown to stimulate the proliferation of thymocytes as well as mature T lymphocytes. We were interested in studying the signal transduction pathway of IL-7 in human T cells isolated from peripheral blood of normal donors and human umbilical cord blood. The IL-7 receptor has been cloned and sequenced but does not appear to contain any sequences with homology to a protein tyrosine kinase domain. We investigated whether a cellular tyrosine kinase activity was induced as an early event in response to IL-7 binding to the IL-7 receptor in T cells.

Upon treatment of purified human T cells with IL-7 (10 ng/ml), gel electrophoresis of cell lysates, and analysis by western blotting using anti-phosphotyrosine antibodies, four proteins with molecular weights of 95 kD (doublet), 105 kD and 130 kD were rapidly tyrosine phosphorylated. Results were similar for T cells from adult peripheral and cord blood.

Another transient, early response to the binding of many growth factors to their respective receptors is the induced expression of primary response genes which encode nuclear proteins such as myc, fos, jun and others. To further elucidate early events in the IL-7 signalling pathway, we examined whether IL-7 induced the expression of primary response genes in mature human T cells. IL-7 treatment of peripheral blood T cells stimulated a three-fold increase in c-myc expression, without requiring protein synthesis. Experiments were also performed with protein kinase inhibitors to characterize the mechanism of induction of c-myc. Results suggest that IL-7 probably does not utilize the protein kinase C pathway for signal transduction.

Non-Receptor Tyrosine Protein Kinases

BZ 217 SIGNAL TRANSDUCTION THROUGH A SYNTHETIC RECEPTOR TYROSINE KINASE COMPOSED OF PDGFR-CD4 AND THE CYTOPLASMIC TYROSINE PROTEIN KINASE LCK,

Dieter Adam[1], Sabine Klages[1], Philippe Bishop[1], Sandeep Mahajan[1], Elisa Eiseman[1], Jaime A. Escobedo[2], Lewis T. Williams[2] and Joseph B. Bolen[1]

[1] Signal Transduction Laboratory, Dept. Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 06543-4000

[2] Univ. Calif. San Francisco, Howard Hughes Med. Inst., San Francisco, CA 94143

We have generated a new "receptor tyrosine kinase" by fusing the extracellular and transmembrane domain of the mouse PDGF receptor to the cytoplasmic domain of CD4 and coexpressing the construct with the murine cytoplasmic tyrosine kinase Lck. In COS cells, both components are associated as shown by coimmunoprecipitation of Lck with PDGFR antibodies.

NMuMG cells, which are mouse mammary gland epithelial cells that lack endogenous PDGF receptor expression were stably transfected with both PDGFR-CD4 and Lck. Expression of the PDGFR-CD4 molecule at the cell surface was confirmed by binding of ^{125}I-PDGF and FACS analysis. Expression of Lck was shown by western blot and immune-complex kinase assays. Co-immunoprecipitation experiments demonstrated the association of PDGFR-CD4 and Lck in the stable lines.

These cells showed an increase in tyrosine phosphorylation of both Lck and cellular proteins when stimulated with PDGF. This response was detectable as early as 1 minute and increases up to 4 hours after stimulation. Lck enzyme activity, as measured by immune-complex kinase assays using enolase as exogenous substrate was increased after PDGF stimulation.

Therefore, a cytoplasmic tyrosine kinase can substitute for the catalytic domain of a receptor tyrosine kinase in inducing tyrosine phosphorylation following binding of the ligand.

BZ 218 FUNCTIONAL CHARACTERIZATION OF THE SH2 DOMAIN OF THE PROTEIN TYROSINE KINASE p56*lck*,

Kurt E. Amrein, Bärbel Panholzer, Nicholas A. Flint, Eric Kitas, Willi Bannwarth and Paul Burn, Pharmaceutical Research-New Technologies, F. Hoffmann-La Roche, CH-4002 Basel, Switzerland

An increasing number of proteins is being recognized to contain non-catalytic domains termed *src* homology 2 and 3 (SH2 and SH3). SH2 domains are believed to be important in mediating protein-protein interactions by recognizing particular phosphotyrosine residues on target polypeptides. The amino acid sequence of the protein tyrosine kinase p56*lck* displays a region with the typical hallmarks of an SH2 domain. To test whether this domain is functional e.g. binds specifically to tyrosine-phosphorylated proteins we expressed *lck*-sequences encoding the *lck*-SH2 domain in *E.coli*. The corresponding recombinant proteins were purified and immobilized on agarose beads. These affinity-matrices bound a subset of tyrosine phosphorylated proteins present in the lysates of transformed NIH3T3 cells expressing *lck*F505 which encodes a constitutively activated mutant form of p56*lck*. Two of the bound proteins were identified as p85, a subunit of PI 3-kinase, and p120-GAP, the GTPase activating protein of p21*ras*. Both proteins bound to the *lck*-SH2 domain-containing polypeptides only when phosphorylated on tyrosine.

It has been proposed that the *src*-SH2 domain inhibits kinase activity of p60*src* by binding to its phosphorylated carboxy-terminus. To test whether a similar mechanism may control p56*lck* activity we synthesized a biotinylated peptide corresponding to the carboxy-terminus of p56*lck* and immobilized it on Streptavidine agarose. The *lck*-SH2 domain bound specifically to the phosphorylated but not to the dephosphorylated peptide.

These results suggest that the *lck*-SH2 domain has a dual function: it can either mediate intermolecular interactions with cellular tyrosine phosphorylated proteins or it can bind the phosphorylated carboxy terminus of p56*lck*, most likely in an intramolecular fashion. We propose that phosphorylation / dephosphorylation of tyrosine 505 might serve as a switch between these two functions.

BZ 219 THE PROPERTIES OF CHIMERAS CONTAINING THE REGULATORY SH2 DOMAINS OF GAP AND PLCγ1 IN THE CONTEXT OF THE ACTIVATED TYROSINE KINASE v-FPS

Deborah H. Anderson[1] and Tony Pawson[2], [1]Cancer Research Unit, Saskatoon Cancer Centre, 20 Campus Drive, Saskatoon, Saskatchewan, Canada S7N 4H4 and [2]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Ave., Toronto, Ontario, Canada M5G 1X5

A conserved noncatalytic domain SH2 (for Src Homology region 2) is located N-terminal to the kinase domain of all cytoplasmic protein tyrosine kinases including that of the v-Fps oncoprotein. Although this domain is not absolutely required for the kinase activity of v-Fps, mutations or deletions within the SH2 domain reduce both its kinase activity and transforming ability. In addition, the SH2 domain of v-Fps has also been shown to be required for the tyrosine phosphorylation of specific cellular proteins of 124 and 62 kDa. These results of these, as well as other experiments have led to the suggestion that SH2 domains form a physical contact with the adjacent kinase domain and serve to modulate its activity, perhaps forming binding sites for substrates critical for transformation and/or protecting kinase domain substrates from subsequent dephosphorylation.

Other proteins, with catalytic activities unrelated to the protein tyrosine kinases have also been found to contain SH2 domains. These include the GTPase activating protein (GAP) of p21^{ras} and phospholipase Cγ1 (PLCγ1), each of which contain two SH2 domains. GAP downregulates p21^{ras} activity and PLCγ1 hydrolyzes phosphatidylinositol-4,5-bisphosphate generating second messengers which mobilize intracellular Ca^{2+} stores and activate Protein Kinase C. The SH2 domains of GAP and PLCγ1 have been shown to be important in mediating protein-protein interactions between those proteins and a distinct set of activated growth factor receptors and well as various tyrosine phosphorylated cytoplasmic proteins.

In order to determine whether the specificity of these protein-protein interactions lies soley within the SH2 regions, chimeric proteins have been constructed in which the v-Fps SH2 domain has been replaced by the SH2 domains of GAP and PLCγ1 in the transforming cytoplasmic tyrosine kinase v-Fps. The tyrosine kinase activity, transforming ability and substrate specificity of these hybrids will be discussed and the contribution to these properties made by various different SH2 domains determined.

BZ 220 FUNCTION AND REGULATION OF THE C-TERMINAL *src*-KINASE, p50^{csk}

Mathias Bergman, Tomas Mustelin*, Manfred Koegl#, Neal Rosen§ and Kari Alitalo Laboratory of Cancer Biology, Depts of Virology and *Pathology, University of Helsinki, 00290 Helsinki, Finland; #EMBL, 6900 Hedelberg, Germany; § Cell Biol. and Genetics, SKI, NY 10021, USA.

We have recently isolated a human cDNA encoding a novel type of cytoplasmic TK, p50^{csk}, with typical TK, SH2 and SH3 domains (1). However, p50^{csk} lacks the conserved autophosphorylation site of tyrosine kinases, has no regulatory C-terminal tyrosyl residue typical for *src*-like kinases, and is not myristylated. We have shown that p50^{csk} phosphorylates exclusively the regulatory Tyr527 of recombinant pp60$^{c\text{-}src}$ and p56^{lck} *in vitro*. The p50^{csk}-mediated phosphorylation strongly suppresses the activity of these TKs *in vitro*. Thus, p50^{csk} functions as a negative regulator of the *src*-family of TKs. Using baculovirus produced, purified p50^{csk} and pp60^{src}, the enzyme-substrate interactions could be studied in more detail. We have also analysed the function of p50^{csk} in signal transduction during T-cell activation, in which p56^{lck} and p59^{fyn} play major roles. Mutations of Tyr527 are known to convert *src*-proteins into transforming oncoproteins. The *csk* tyrosine kinase may thus play a crucial role in the regulation of normal cell growth and may be regarded as a tumor suppressor protein.

1. Bergman, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N.A., Amrein, K.E., Autero, M., Burn, P. and Alitalo, K.: The human p50*csk* tyrosine kinase phosphorylates p56*lck* at Tyr-505 and down-regulates its catalytic activity. ***EMBO J.*** 11: 2919-2924, 1992.

BZ 221 KIZ-1, A VERY UNUSUAL PROTEIN WITH KINASE AND ZINC FINGER DOMAINS.

Ora Bernard and Soula Ganiatsas: The Walter and Eliza Hall Institute of Medical Research, P.O. Royal Melbourne Hospital, Parkville, Victoria 3050, Australia

The olfactory epithelium is the only neuronal tissue capable of generating new neurons during adult life and therefore it must express genes responsible for this phenomenon. Therefore we have used immortalized olfactory epithelial cells as a source of mRNA for the isolation of new protein tyrosine kinases (PTK) by PCR using oligonucleotides to the conserved regions in the catalytic domain of the PTK. The full size cDNA clone of one of these PCR products was isolated and sequenced. This cDNA, designated Kiz-1, encodes a protein containing two main domains. The N-terminal domain contains a cystein-histidine rich region previously described as the zinc finger domain of proteins belonging to the LIM family and the C-terminus contains a kinase domain. Northern blot analysis indicated that Kiz-1 is expressed mainly in the brain of adult mice and also in the developing brain during neuronal differentiation, reaching its highest level of expression at embryonic day 16. Kiz-1 is also expressed in all the cell lines examined regardless of their origin. Immunohistochemistry studies on adult mouse brain with anti-Kiz-1 antibodies demonstrated that Kiz-1 is expressed exclusively in neurons and not in astrocytes or oligodendrocytes. Interestingly, in the developing embryos Kiz-1 is expressed in all tissues. These results indicated that Kiz-1 is expressed in dividing cells and in neurons and therefore may have a role in cellular growth and maintenance of neurons. In COS cells transfected with Kiz-1 cDNA and in the immortalised olfactory epithelial cells, Kiz-1 was found mainly in the cytoplasm. However in neurons of the adult brain it is found also in the nucleus.

BZ 222 P56lck RESPONSE TO INTERLEUKIN-2 IN HUMAN NATURAL KILLER CELLS, Jacques Bertoglio and Isabelle Vitté-Mony, INSERM U333, Institut Gustave Roussy, 94805 Villejuif, FRANCE.

The T-cell specific tyrosine kinase p56lck is expressed in human natural killer (NK) cells. Whether p56lck is critically involved in interleukin-2 (IL-2) signal transduction remains a matter of controversy. We have addressed this question by studying p56 kinase activity and shift in electrophoretic mobility in response to IL-2, in human NK cell lines expressing high affinity IL-2 receptors and in freshly purified NK cells that only express the intermediate affinity IL-2 receptor ß-chain.
Stimulation of NK cell lines with IL-2 induces a time and dose dependent increase in p56lck kinase activity, as measured by autophosphorylation, and phosphorylation of rabbit enolase, as well as a typical electrophoretic mobility shift. When these cells are stimulated in the presence of an anti-Tac antibody (BB10 Mab), which effectively blocks the binding of IL-2 to its high affinity receptor, but not to the intermediate affinity ß-chain, a similar shift is observed, which however requires an higher IL-2 concentration, and displays a delayed kinetics. These changes probably reflect differences in the parameters of interaction of IL-2 with its high and intermediate affinity receptors.
However, no modifications of p56lck can be observed in freshly purified NK cells in response to IL-2, despite the fact that resting NK cells express the IL-2 receptor ß chain, and are readily activated by IL-2 which induces a dramatic increase of their cytolytic activity.
Our current interpretation for these discrepancies is two-fold : 1) p56lck activation may not be required for IL-2-induced increase in cytotoxic activity as opposed to IL-2-induced proliferation, and 2) p56lck coupling to the IL-2 receptor ß chain may depend upon additional signals or additional components of the IL-2 receptor which are missing in resting NK cells.

BZ 223 CHARACTERIZATION OF YEAST MCK1 DUAL-SPECIFICITY PROTEIN KINASE, Derrick Brazill, Moon Young Lim, David Dailey, Jeremy Thorner, G. S. Martin. Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720

The purified MCK1 kinase from the yeast *Saccharomyces cerevisiae* behaves as a dual-specificity kinase which autophosphorylates on tyrosine and serine residues and phosphorylates several yeast proteins on serine and/or threonine residues. *In vivo*, the MCK1 kinase activity is required for its tyrosine autophosphorylation.

The MCK1 gene product appears to be involved in the maintenance of microtubule structure. Δmck1 mutant cells are hypersensitive to sublethal concentrations of benomyl, a microtubule destablizing compound. These cells show normal growth in the absence of benomyl, but arrest in its presence. Immunofluorescence analysis demonstrates that Δmck1 cells in 10 µg/ml benomyl have a drastically increased number of microtubule defects when compared to wild type cells under the same conditions.

Several *in vitro* protein substrates of the MCK1 kinase have been identified. Myelin basic protein and microtubule associated protein II are phosphorylated on serine residues and tau protein is phosphorylated on serine and threonine residues. The conditions that are optimal for autophosphorylation and the conditions that are optimal for the phosphorylation of exogenous substrates are distinct.

An MCK1 homologue has been cloned from *Schizosaccharomyces pombe* by complementation of the *S. cerevisiae* null allele. The cloned gene produces a protein that cross reacts with the anti-MCK1 polyclonal antibody.

BZ 224 THE SRC FAMILY OF TYROSINE KINASES: REGULATION OF ACTIVITY AND INVOLVEMENT IN TRANSFORMATION BY POLYOMA VIRUSES, Leonardo Brizuela, Lucia Olcese, Margaret Jones and Sara A. Courtneidge. European Molecular Biology Laboratory, 6900 Heidelberg, F.R. Germany.

The transforming protein of mouse polyomavirus, middle T antigen, forms complexes with Src family protein tyrosine kinases, phosphatidylinositol 3-kinase, and the serine/threonine phosphatase PP2A. Genetic analysis shows that the ability of mT to associate with cellular proteins is required in order for it to be transforming. The mouse middle T antigen associates predominantly with Src and Yes, and only very poorly with Fyn. We have recently characterised a new middle T antigen (from the lymphoma-causing hamster polyomavirus) and shown that, like its mouse counterpart, it associates with tyrosine kinase, lipid kinase, and serine/threonine phosphatase activity. However, the hamster middle T antigen associates exclusively with Fyn in fibroblasts, not with Src or Yes. We have created a number of chimeric molecules in order to map the determinants that dictate this binding specificity. In addition, a comparison of the phosphoproteins in cells transformed with these two middle T antigens has allowed us to demonstrate differences in substrate specificity between the members of the Src family. We have also now begun to use these two middle T antigens to study the involvement of Src family kinases in signal transduction in lymphocytes.

BZ 225 **EXPRESSION OF THE *vav* PROTO-ONCOGENE IS CONFINED TO THE EMBRYONIC AND ADULT HAEMATOPOIETIC SYSTEM.** Xosé R. Bustelo and Mariano Barbacid, Department of Molecular Biology, Bristol-Myers Squibb Pharm. Research Inst., Princeton, NJ, 08543.

The product of the *vav* proto-oncogene, p95^{vav}, is a SH2/SH3-containing protein which exhibits a series of structural motifs including a HLH/leucine zipper-like domain, a cysteine rich region and a domain related to the Dbl GDP/GTP exchange factor. Previous studies have shown that p95^{vav} ectopically expressed in NIH3T3 cells becomes associated with and is phosphorylated by ligand-activated PDGF and EGF receptors. More importantly, engagement of the T-cell TCR/CD4 complex and the mast-cell receptor leads to the rapid phosphorylation of the endogenous p95^{vav} on tyrosine residues. Similar results have been recently obtained in c-Kit-expressing cells upon addition of *Steel* factor and in B-cells following activation of their IgM receptors. Previous studies using cell lines have indicated that expression of the *vav* proto-oncogene may be restricted to haematopoietic cells. Now, we have extended these observations by investigating the expression of the *vav* gene during mouse development using *in situ* hybridization. *Vav* gene transcripts were first detected in the liver of 11.5 day embryos where expression was confined to differentiating haematopoietic cells. In later stages of development, *vav* expression become down-regulated in the liver and sequentially activated in thymus, spleen and bone marrow. In the thymus, *vav* expression occurs mainly in the cortical zone where immature thymocytes undergo differentiation. Immunocytochemistry studies show that p95^{vav} is preferentially expressed in the cytoplasm. After birth, *vav* expression remained restricted to haematopoietic tissues with the exception of the ameloblast layer of the tooth enamel organ. The strict correlation of *vav* gene expression with the onset and development of the haematopoietic system, along with its rapid phosphorylation following engagement of multiple haematopoietic receptors, suggests an important role for the Vav protein in haematopoietic cell signalling.

BZ 226 REGULATION OF ADIPOCYTE DIFFERENTIATION THROUGH SIGNALLING PATHWAYS MODULATED BY POLYOMA MIDDLE T ANTIGEN. Van Cherington[1-4], Cynthia Higgins[1], and Mitch Hardenbrook[3,4], Departments of Physiology[1], Anatomy and Cell Biology[2], and Pathology[3], Tufts Univ. Schl of Med. and New England Medical Center Hospital[4], Boston, MA. 02111.

The murine preadipocyte cell line, 3T3-L1, terminally differentiates into functional adipocytes, following treatment with glucocorticoid, when cultured at confluence in medium containing fetal calf serum and insulin. Mitogenic signalling pathways regulated by distinct growth factors and hormones have opposing regulatory roles in these cells. In order to determine the role of multiple signalling enzymes in the positive and negative regulation of differentiation we have expressed Polyomavirus middle T antigen (PyMT) in 3T3-L1 preadipocytes. PyMT alters cellular signal transduction by associating with and activating phosphatidylinositol-3'-kinase (PI3K) and c-src or related non-receptor tyrosine kinases. PyMT also binds to the 36KDa and 63KDa components of the protein phosphatase 2A (PP2A) complex. A genetic analysis of PyMT in 3T3-L1 preadipocytes reveals distinct roles for PyMT targets in adipocyte differentiation.

Constitutive expression of wild-type PyMT in 3T3-L1 preadipocytes blocks adipose differentiation prior to induction of differentiation-dependent mRNA, glycerophosphate dehydrogenase activity (GPD), and triglyceride production. The PyMT mutant dl23, which fails to associate with and activate cellular PI3K, does not suppress differentiation but permits insulin-independent adipose differentiation. Preadipocytes expressing PyMT mutant Ng59, which does not associate with PI3K, c-src, or PP2A, express insulin-dependent differentiation like parental 3T3-L1 cells. These results implicate the PI3K association with blocking differentiation and the src-family tyrosine kinase and/or PP2A association with insulin-independence. Nanomolar okadaic acid stimulates insulin-independent differentiation of 3T3-L1 cells. This is consistent with a role for PP2A modulation in insulin-regulated adipogenesis.

BZ 227 EXPRESSION OF AN LCK DELETION MUTANT CONFERS ENHANCED CD4 SIGNALLING CAPABILITY, Tassie L. Collins and Steven J. Burakoff, Division of Pediatric Oncology, Dana Farber Cancer Institute, Boston, MA 02115

The T cell surface glycoprotein CD4 acts as a co-receptor in T cell activation, enhancing stimulation via the TCR/CD3 complex. This co-receptor function requires the association between CD4 and the protein tyrosine kinase, p56^{lck}, since disruption of the CD4:p56^{lck} complex abrogates CD4-mediated IL-2 production, and overexpression of p56^{lck} results in heightened antigen responsiveness. However, the exact nature of the role of p56^{lck} in CD4-mediated signal transduction remains unclear. Here we demonstrate that overexpression of an lck mutant lacking the kinase domain (lckΔ^{176}) in a T cell hybridoma results in a significant increase in CD4-dependent IL-2 production. Cells overexpressing lckΔ^{176} demonstrate enhanced association between CD4 and the TCR/CD3 complex during stimulation via anti-CD3 antibody, as demonstrated by fluorescence resonance energy transfer. Finally, immunoprecipitation of CD4 from cells expressing lckΔ^{176} reveals a significant increase in associated GTP-binding activity. These data suggest that lck has multiple roles in antigen response independent of kinase activity. Additionally, the data suggest that the GTP-binding protein associated with the CD4:p56^{lck} complex is involved in CD4-mediated enhancement of antigen response.

BZ 228 A BRUSH BORDER NON-RECEPTOR TYROSINE KINASE STIMULATES INTESTINAL NaCl ABSORPTION AND BRUSH BORDER Na^+/H^+ EXCHANGE. Mark Donowitz, Michael E. Cohen, Jami Montgomery, Susan Walker, Departments of Medicine and Physiology, GI Unit, Johns Hopkins University School of Medicine, Baltimore, MD 21205

The drug genistein was used to define a role for tyr kinases in regulation of basal and stimulated neutral NaCl absorption in rabbit ileum, studied with the Ussing chamber/voltage clamp technique and by direct addition to brush border vesicles made from ileal villus cells. Genistein added to the ileal mucosal surface inhibited neutral NaCl absorption and added to vesicles inhibited brush border Na^+/H^+ exchange. In contrast, other transport processes were not affected by genistein including D-glucose stimulated Na^+ uptake, Na^+ stimulated glucose uptake, and glucose and Na^+ equilibrium volumes in vesicles. Furthermore, these effects were not duplicated by genistin, a drug with similar structure to genistein but lacking tyr kinase inhibiting properties. Serosal but not mucosal EGF stimulated NaCl absorption. Mucosal genistein but not genistin also altered second messenger regulation of neutral NaCl absorption, inhibiting the effect of Ca^{2+} ionophore A23187 and of serosal EGF but not affecting the transport changes caused by 8-Br-cAMP. In contrast, the Cl secretory effects for all three agents, A23187, EGF, and 8-Br-cAMP, were all inhibited by mucosal genistein. These results demonstrate that genistein is acting as a specific tyr kinase inhibitor in these studies since its effects were not duplicated by a compound with similar non-specific effects but without the ability to affect tyr kinases. These results indicate that: 1) a tyr kinase is involved in basally inhibiting ileal neutral NaCl absorption and brush border Na^+/H^+ exchange; 2) brush border tyr kinase is involved in the ability of Ca^{2+} ionophore A23187 to inhibit neutral NaCl absorption but is not involved in the transport effects of cAMP; 3) EGF stimulates NaCl absorption by an effect exerted from the serosal surface but the effect also involves a brush border tyr kinase. This study establishes that tyr kinase(s) acting over short time periods is involved in stimulation of neutral NaCl absorption and brush border Na^+/H^+ exchange and represents the first example of a brush border tyr kinase being involved in short-term signal transduction in epithelial cells.

BZ 229 *HCK* TYROSINE KINASE ACTIVITY MODULATES TUMOR NECROSIS FACTOR PRODUCTION IN THE MURINE MACROPHAGE CELL LINE BAC1.2F5

B. Keith English[1], James N. Ihle[2], Angela Myracle[1], and Taolin Yi[2], [1]Dept. of Pediatrics, The University of Tennessee, Memphis, and [2]Dept. of Biochemistry, St. Jude Children's Research Hospital.

The *hck* gene, a member of the *src* family of tyrosine kinases, is expressed in cells of the monocyte/macrophage and granulocyte lineages. In human monocyte/macrophages, *hck* expression increases with differentiation and is augmented by activation stimuli. To examine the potential role of *hck* in macrophage activation, we used the CSF-1 dependent murine macrophage-like cell line BAC1.25, which expresses *hck* mRNA, $p59^{hck}$ protein and *hck* kinase activity. In BAC1.2F5 cells, *hck* mRNA and protein levels increased within hours of exposure of quiescent cells to growth factors (e.g. CSF-1, GM-CSF) and activating stimuli (e.g. LPS), while *hck* kinase activity was augmented within minutes of exposure to these stimuli. We found that manipulation of the level of *hck* expression altered the responsiveness of these cells to activation by LPS but did not affect survival or proliferation. BAC1.2F5 cells expressing an activated mutant of *hck* ($p59^{hckF501}$) accumulated 5-50 fold more TNF mRNA and secreted 3-4 fold more TNF protein in response to LPS than did parental cells or cells expressing vector alone or a kinase-defective mutant of *hck* ($p59^{hckE269}$). In contrast, exposure of BAC1.2F5 cells to phosphorothioated *hck* antisense oligonucleotides resulted in a 3-4 fold reduction in *hck* protein levels and *hck* kinase activity and led to a 3-4 fold reduction in TNF mRNA accumulation and TNF secretion by these cells in response to LPS. The reduction in TNF production was comparable to that observed after pretreatment of BAC1.2F5 cells with the tyrosine kinase inhibitor, herbimycin A. Together, these data suggest an important role for *hck* in signaling pathways central to macrophage activation and cytokine gene expression.

BZ 230 IL-2-DEPENDENT ACTIVATION OF RECEPTOR SPECIFIC AND MITOGENIC TYROSINE KINASE PATHWAYS: INCREASED ACTIVITY OF A p97 MITOGENIC PATHWAY IN HTLV-1 TRANSFORMED T CELLS, (1)Evans, G.A., (2)Kirken, R.A., and (2)Farrar, W.L., (1)Biological Carcinogenesis and Develpoment Program, Program Resources Inc./DynCorp, and (2)Cytokine Mechanisms Section, Laboratory of Molecular Immunnoregulation, Biological Response Modifiers Program, National Cancer Institute, Frederick Cancer Research and Development Center, Frederick, MD 21702.

An early event associated with interleukin-2 (IL-2) signal transduction is a rapid increase in tyrosine kinase activity, made evident by increased tyrosine phosphorylation of several key signal transduction substrates. Using the IL-2 responsive cell line YT, we show that the principle tyrosine kinase substrates detected in response to IL-2 by anti-phosphotyrosine immunoblotting of total cellular proteins or anti-phosphotyrosine immunoprecipitates are of 116 and 97 kDa. pp116 shows the most rapid kinetics with maximal phosphorylation occuring within 1 minute in response to IL-2 while pp97 is maximally phosphorylated by 10 min.s. The addition of pervanadate to serum deprived YT cells results in rapid phosphorylation of pp97 followed by cell cycle progression and mitogenesis without equivalent phosphorylation of pp116. This suggests that the phosphorylation of pp97 is associated with mitogenesis while pp116 phosphorylation is strictly associated with early IL-2 signal transduction leading toward eventual pp97 phosphorylation and mitogenesis. Analysis of HTLV-1 transformed human T cells shows a dramatic increase of IL-2 responsive pp97 compared to that seen in normal human T cells or the YT cell line. Pervanadate treatment of these cells also results in a rapid and linear, preferential increase in pp97 phosphorylation. Together this data suggests that phosphorylation in response to IL-2 can be divided into strictly ligand/receptor associated events (pp116 phosphorylation) and those events which converge on mitogenic pathways (pp97 phosphorylation) and further that an increase in the pp97 mitogenic pathway is associated with HTLV-1 transformation.

BZ 231 v-Src-INDUCED INTRACELLULAR SIGNALING INVOLVES AT LEAST THREE DISTINCT GTPASE-DEPENDENT STEPS

David A. Foster, Konstantina Alexandropoulos, Sajjad A. Qureshi, Hong Jiang and Jianguo Song, *The Institute for Biomolecular Structure and Function and The Department of Biological Sciences, The Hunter College of The City University of New York, 695 Park Avenue, New York, NY 10021.*

v-Src induces gene expression under the control of both serum response elements (SREs) TPA response elements (TREs). The induction of TRE-mediated gene expression by v-Src is dependent upon protein kinase C (PKC); whereas, the induction of SRE-mediated gene expression is independent of PKC. Both v-Src-induced TRE- and SRE-mediated gene expression were sensitive to a dominant-negative HaRas mutant. An activated derivative of HaRas, v-HaRas, also activated both TRE- SRE-mediated transcription. v-Src-induced TRE-mediated gene expression was sensitive to depleting cells of PKC, whereas, v-HaRas-induced TRE-mediated transcription was insensitive to PKC depletion suggesting that HaRas functions downstream from PKC in v-Src-induced TRE-mediated gene expression. Consistent with this hypothesis, the induction of TRE-mediated gene expression by phorbol esters that activate PKC directly, was also blocked by the dominant-negative HaRas mutant. Thus, v-Src-induced activation of TRE-mediated gene expression is via an intracellular signalling mechanism that is dependent upon both PKC and HaRas where HaRas functions downstream from PKC. Since v-Src-induced SRE-mediated gene expression is independent of PKC, theses data suggest that v-Src activates HaRas via to distinct mechanisms.

v-Src activates PKC via a phospholipase D PLD and a phosphatidate phosphatase (1,2) which together lead to the production of diacylglycerol, the physiological activator of PKC. The activation of PLD activity by v-Src is sensitive to GDPßS, which blocks GTPases. Consistent with the involvement of a GTPase in the activation of PLD activity, GTPγS, a non-hydrolysable analogue of GTP, increased PLD activity in the parental BALB/c 3T3 cells. Thus, there is likely a third GTPase that is upstream from PKC in v-Src-induced intracellular signals.

1) Song, J., Pfeffer, L.M, and Foster, D.A. (1991). *Mol. Cell. Biol.* 11: 4903-4908.
2) Song, J., and Foster, D.A. Manuscript submitted for publication.

BZ 232 MUTATIONS OF THE cABL LAST EXON LEAD TO ITS ONCOGENIC ACTIVATION, Andrei Goga[2], Jami McLaughlin[1,2], Ann Marie Pendergast[1], Kalindi Parmar[4] Naomi Rosenberg[4,5], Owen N. Witte[1,2,3]
Department of Microbiology[1] and Molecular Biology Institute[2] and Howard Hughes Medical Institute[3]. University of California, Los Angeles.
Departments of Pathology[4] and Molecular Biology and Microbiology Tufts University School of Medicine[5]

cABL is a non-receptor tyrosine kinase which shares regions of homology with members of the *src*-related gene family. It differs from other members of this family by containing a large last exon. Oncogenic forms of cABL have previously been shown to contain mutations within the first exon or src homology (SH) regions. Replacement of the cABL first exon plus SH3 region with viral gag gene sequences has been shown to lead to oncogenic activation in murine v-ABL. The Philadelphia Chromosome results from translocations replacing the amino terminal exon of cABL with a portion of the BCR gene. These mutants of cABL have an activated tyrosine kinase and contain phosphotyrosine. The last exon of cABL has been shown to be crucial for proper growth and development in both mice and fruit flies. We describe the isolation and characterization of a novel in-frame deletion of the last exon of cABL that leads to a transforming phenotype in rodent fibroblast cells. cABL mutants containing several smaller deletions within this region were created which retained the transforming phenotype. The mechanism of activation in this series of mutants appears to differ from previously described activating mutations. The presence of phosphotyrosine on cABL is not detected in these mutants. They are also unable to rescue lymphoid cells transformed with a temperature sensitive form of vABL at the non-permissive temperature. The last exon plays an important role in the regulation of cABL function and mutations in this region can lead to cABL oncogenic activation.

BZ 233 *FYN TYROSINE KINASE IS SPECIFICALLY REQUIRED FOR SYNAPTIC PLASTICITY AND LEARNING AND MEMORY,* Seth Grant, Thomas O'Dell, Kevin Karl, Paul Stein*, Philippe Soriano* and Eric Kandel. HHMI, Ctr. Neurobiol. & Behav., Columbia P & S, NY, NY 10032; HHMI, Baylor Coll. Med. Houston, TX 77030*

We have used a genetic approach to identify molecules involved with the synaptic plasticity that underlies vertebrate learning and memory. Long-term potentiation (LTP) is the prolonged enhancement of synaptic strength resulting from a brief period of increased neuronal activity. The induction of LTP requires the activation of the NMDA-subtype glutamate receptor, calcium influx and activation of both serine/threonine and tyrosine kinases. Because the tyrosine kinase inhibitors that block LTP (O'Dell et al, 1991) do not discriminate between tyrosine kinases, we sought to identify specific kinases involved with LTP, by testing mice harboring mutations in non-receptor tyrosine kinase genes. We examined mice carrying homozygous null-mutations in *fyn, src, yes* and *abl*, which are expressed in the hippocampus.

There was a blunting of LTP in mice lacking *fyn* but not *src, yes* or *abl* tyrosine kinase expression. This defect was selective for LTP since synaptic transmission and other forms of synaptic plasticity were intact. Since the hippocampus is required for spatial learning, and LTP is a cellular model of learning, we tested the ability of *fyn*$^-$ mice to learn a spatial task. The *fyn*$^-$ mice showed impaired spatial learning in the Morris water maze supporting a functional link between LTP and learning.

The *fyn* gene is also necessary for the normal development of the hippocampus since there are anatomical abnormalities in the CA1 and CA3 regions and the dentate gyrus. The cell body layer of the CA3 and dentate gyrus undulates suggesting increased cell number and the apical dendrites of the CA1 region appear disorganized. Despite these morphological changes, the CA3 to CA1 synapses show normal synaptic transmission and simple forms of plasticity including paired-pulse facilitation and post-tetanic potentiation. Consistent with the expression of *fyn* at synapses, this data suggests that *fyn* plays a role in regulating synaptic plasticity both in the developing and mature synapse.

O'Dell, T.J., Kandel, E.R., and Grant, S.G.N. (1991) Long-term potentiation in the hippocampus is blocked by tyrosine kinase inhibitors. *Nature*,353:558-560.

BZ 234 V-SRC TRANSFORMATION OF MOUSE FIROBLASTS IS REVERSED BY OKADAIC ACID: Ruchika W. Gupta, Cecil K. Joseph and David A. Foster; The Institute for Biomolecular Structure and Function, and The Department of Biolgical Sciences, The Hunter College of the City University of New York, 695 Park Avenue, New York, NY 10021.

Okadaic acid (OA) is a potent inhibitor of the serine/threonine phosphatases 1 (PP1) and 2A (PP2A). Transformation of NIH/3T3 fibroblasts by an activated c-Raf-1 can be reversed by OA (1). We have demonstrated that c-Raf-1 serves as a signal intermediary in the activation of the mitogen-responsive transcription factor Egr-1 by v-Src (2). We therefore examined the effect of OA upon several transformation-related phenotypes in v-Src-transformed Balb/c 3T3 fibroblasts. Addition of OA to v-Src-transformed mouse fibroblasts led to a flat, non-transformed morphology, decreased colony formation in soft agar, and increased fibronectin levels. v-Src-induced increases in [^{3}H]-thymidine incorporation and the rate of proliferation of v-Src-transformed cells were also inhibited by OA. The state of phosphorylation and level of the v-Src protein were unaffected by okadaic acid. These data suggest that a protein phosphatase 1 or 2A may be required for the transformation of cells by v-Src. We have generated stable cell lines of v-Src-transformed mouse fibroblasts expressing antisense PP2A. Results of the characterization of the cell lines and the involvement of PP2A in v-Src signaling will be presented.

1. Sakai, R. et al. *(1989) Proc. Natl. Acad. Sci. USA* **86**, 9946-9950.

2. Qureshi, S. A. et al. (1991) *J. Biol. Chem.* **266**, 20594-20597.

BZ 235 MUTATIONAL ANALYSIS OF THE FOCAL ADHESION KINASE. Jeffrey D. Hildebrand, Michael D. Schaller, Cheryl A. Borgman, Bradley C. Cobb, and J.Thomas Parsons, Department of Microbiology and Cancer Center, University of Virginia School of Medicine, Charlottesville, Va. 22908

The integrin family of transmembrane proteins function as receptors for components of the extracellular matrix and simultaneously interact with components of the cytoskeleton and focal adhesions eg. α-actinin and talin. The intergins play important roles in cellular processes such as cell adhesion, migration, development, differentiation, and metastasis. Integrin-ECM interactions induce intracellular changes in cytoskeletal organization, pH, calcium concentrations, and tyrosine phosphorylation of cellular proteins. The Focal Adhesion Kinase, pp125^{FAK}, colocalizes to focal adhesions with intergins. Stimulation of integrins induces an increase in the phosphotyrosine content of pp125^{FAK}, which correlates with an increase in pp125^{FAK} kinase activity measure *in vitro*. Thus, pp125^{FAK} may play a critical role in integrin-mediated cellular events. The phosphotyrosine content of pp125^{FAK} increases in CE cells transformed by pp60^{src}. pp125^{FAK} complexes with oncogenic forms of pp60^{src}, and this interaction is dependent upon the integrity of the src SH2 domain. *src* mutants that do not bind pp125^{FAK} are non-transforming, thus pp125^{FAK} may play a role in *src* oncogenicity. Mutational analysis of pp125^{FAK} has been initiated to investigate the biology of pp125^{FAK} in integrin signaling and *src* transformation. An epitope tag has been employed in order to define the subcellular localization of exogenously expressed mutants. Results indicate that the carboxyl terminus is required for pp125^{FAK} localization to focal adhesions. When overexpressed, neither wild-type pp125^{FAK} nor pp125^{FAK} deletion or point mutants induce an observable effect on cell morphology. Efforts are being made to identify the role of pp125^{FAK} in integrin-mediated cell adhesion and migration by using CE cells overexpressing wild-type pp125^{FAK} or a kinase defective mutant (potential dominant negative) to augment or perturb normal integrin-mediated events. Similar strategies are planned for investigating the role of pp125^{FAK} in *src* transformation.

BZ 236 EFFECT OF TYRPHOSTINS, INHIBITORS OF PROTEIN TYROSINE KINASES, ON TYROSINE KINASE ACTIVITY OF ACTIVATED SRC PROTEIN IN NIH3T3 CELLS, A. Jacquemin-Sablon, W.K. Agbotounou, A. Levitzki* and J. Pierre, U140 INSERM and URA 147 CNRS Institut Gustave Roussy, 94800 Villejuif, France; *Institute of Life Sciences, The Hebrew University of Jerusalem, Givat Ram, 91904 Jerusalem, Israel.

NIH3T3 cells have been transfected with c-src mutant gene, F527, encoding for pp60$^{c\text{-}src\text{-}F527}$. These cells are endowed with a high tyrosine kinase activity, evidenced by the elevated phosphotyrosine content of cellular substrates. This elevated cellular substrates phosphorylation is correlated with cell morphology transformation.

Tyrphostins, synthetic compounds, have been described as in vitro and in vivo Epidermal growth factor-Receptor tyrosine kinase inhibitors (Lyall et al., J.Biol.Chem.266,14503-14509). Using the c-src transfected cells, we show that tyrphostins also inhibit pp60$^{c\text{-}srcF527}$ tyrosine kinase activity in intact cells and in vitro. In intact cells, optimal inhibition was observed after 24 h exposure at a concentration of 100 uM, leading to 65 to 85% inhibition of the phosphorylation of the three major substrates: p120, p85 and p62. The same tyrphostins could also reverse the transformed morphology of pp60$^{c\text{-}srcF527}$ expressing cells at 10 to 100 uM after 72 h exposure. Tyrphostins also efficiently inhibited the kinase activity of pp60$^{c\text{-}srcF527}$ in vitro. Our results indicate that tyrphostins reversed the transformed morphology of the pp60$^{c\text{-}srcF527}$ transfected cells, while directly inhibiting the pp60$^{c\text{-}srcF527}$ kinase activity in cells.

BZ 237 **ACTIVATED SRC KINASE PHOSPHORYLATES TYR 457 OF BOVINE GAP IN VITRO AND THE CORRESPONDING RESIDUE OF RAT GAP IN VIVO,** Richard Jove and Soochul Park, Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109

Previous studies demonstrated that Ras GTPase-activating protein (GAP) is phosphorylated on tyrosine in cells transformed by v-Src, suggesting that GAP may provide a biochemical link between v-Src and Ras signaling pathways. To further investigate molecular interactions between Src kinases and GAP, we constructed recombinant baculovirus vectors that direct expression of v-Src, chicken c-Src and bovine GAP in infected Sf9 insect cells. In vitro reconstitution experiments using baculovirus-expressed proteins demonstrate that v-Src and c-Src specifically associate in complexes with GAP. Phosphopeptide mapping analysis revealed that both of the Src kinases phosphorylate GAP at one major site and one minor site in reconstituted complexes in vitro. Significantly, the major site of GAP phosphorylation in vitro, Tyr 457 of bovine GAP, is also the major site of in vivo tyrosine phosphorylation of GAP in rat fibroblasts transformed by v-Src. Our results demonstrate that activated Src kinase associates with GAP and induces phosphorylation of the same tyrosine residue of GAP in vitro and in vivo, suggesting that GAP is a direct substrate of Src kinases in vivo. Because epidermal growth factor receptor phosphorylates the equivalent tyrosine residue in human GAP (Tyr 460), these findings suggest that specific phosphorylation of GAP at this site by oncogenic and receptor kinases may contribute to regulation of mitogenic Ras signaling pathways. Experiments are in progress to define the physiologic significance of this tyrosine phosphorylation in GAP.

BZ 238 **MOLECULAR CLONING AND ANALYSIS OF cDNA ENCODING THE MURINE *c-yes* TYROSINE PROTEIN KINASE,**

Sabine Klages[1], Dieter Adam[1], Elisa Eiseman[1], Joseph Fargnoli,[1] Susan M. Dymecki[2], Stephen V. Desiderio[2], and Joseph B. Bolen[1]

[1] Signal Transduction Laboratory, Dept. Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 06543-4000

[2] Department of Molecular Biology and Genetics, Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The cellular *yes* (*c-yes*) gene is a member of the class of proto-oncogenes which encode non-receptor tyrosine protein kinases. We have isolated cDNAs which encode the murine *c-yes* gene product and analysed the nucleotide sequence of the murine *c-yes* cDNA clones. The reading frame encodes a protein of 541 amino acids with a calculated molecular mass of 60.63 kilodaltons which is reactive with anti-Yes antisera and possesses protein kinase activity. Comparison of the predicted protein encoded by the murine *c-yes* gene to *c-yes* proteins from other species revealed 96% sequence identity to human Yes, 91% to chicken Yes, and 89% to Xenopus laevis Yes. The greatest degree of variation from species to species was found in the unique domain (amino acids 8 to 92) of the proteins. Within the unique domain stronger sequence identity was observed in the carboxy-terminal end compared to the more amino-terminal portion. Alignment with other murine src-family members demonstrates that Yes represents a typical member of this family. Comparison of the 5' untranslated region of the murine c-yes cDNA with the human c-yes promoter region reveals several sequence similarities including the presence of common GC-box like motifs. These observations suggest that the human and mouse c-yes genes possess related transcriptional regulation mechanisms.

BZ 239A DOMINANT-NEGATIVE TRANSGENE DEFINES A ROLE FOR p56^{lck} IN THYMOPOIESIS, Steven D. Levin, Stephen J. Anderson, Katherine A. Forbush, and Roger M. Perlmutter, Departments of Biochemistry and Immunology and the Howard Hughes Medical Institute, University of Washington, Seattle, Washington, 98195.

The lymphocyte-specific protein tyrosine kinase p56^{lck} participates in T cell signaling through functional interactions with components of the T cell antigen receptor complex and the interleukin-2 receptor. Additional insight into the function of p56^{lck} has now been obtained through the generation of transgenic animals expressing high levels of a catalytically inactive form of this kinase (p56^{lckR273}). Mice bearing the *lck*R273 transgene manifested a severe defect in the production of virtually all T lymphocytes. Those exceptional cells that escaped the effects of the *lck*R273 transgene were confined primarily to the T cell subset that expresses γ/∂ T cell receptors. Remarkably, construction of a dose-response curve for the effects of the *lck*R273 transgene revealed that developmental arrest of thymocytes occurred at a discrete stage in the normal T cell maturation pathway, corresponding to a point at which thymoblasts ordinarily begin a series of mitotic divisions that result in expansion and maturation. These results suggest that p56^{lck} normally regulates T cell production by metering the replicative potential of immature thymoblasts. In addition, a comparison of the effects of a homozygous *lck* gene disruption (Molina et al., 1992, Nature 357, 161-4) and the dominant negative transgene suggests that while both produce a block in thymocyte development, the dominant negative mutation more effectively abrogates T cell maturation. Our results suggest that under certain circumstances dominant negative mutations provide a superior means of dissecting signaling pathways in mammalian cells.

BZ 240 BINDING CHARACTERISTICS OF HUMAN C-SRC SH2 AND SH3 DOMAINS TO TYROSINE PHOSPHORYLATED PEPTIDES

Michael A. Luther, Byron Ellis, Derril Willard, Marc Rodriguez, Judd Berman, Tim Lansing, Deirdre Luttrell, and Tona Gilmer, Departments of Structural and Biophysical Chemistry and Cell Biology, Glaxo Research Institute, Research Triangle Park, NC 27709

Recent evidence suggests that proteins which contain the src homology regions SH2 and SH3 play key roles in signal transduction. These structural motifs have been implicated in mediating protein:protein interactions. While SH2 domains have been shown to bind tyrosine phosphorylated proteins, little is known about the function of SH3 domains, although they may be involved in interactions with the cytoskeleton. In order to define the binding specificity and characteristics of these domains, we have expressed, purified and characterized SH2, SH3, and SH3-SH2 of $pp60^{c\text{-}src}$ as free and glutathione-S-transferase (GST) fusion proteins. Using BIAcore technology we have quantitated the specific binding of affinity purified SH2 and SH3 domains of $pp60^{c\text{-}src}$ to immobilized tyrosine phosphorylated and nonphosphorylated peptides. The peptides used in these binding studies correspond to the residues surrounding the C-terminus of $pp60^{src}$ and to phosphopeptides from the p125 Focal Adhesion Kinase (FAK). GST and the GST-SH3 fusion protein were unable to bind to either immobilized phosphorylated or nonphosphorylated peptides. In contrast, the GST-SH2 and GST-SH3-SH2 domains bound to their target tyrosine phosphorylated peptide as determined from both the k_{on}/k_{off} and under steady-state conditions. No binding was observed to immobilized nonphosphorylated peptide. Free phosphopeptide incubated with GST-SH2 or GST-SH3-SH2 was able to compete for binding with the immobilized peptide, whereas the nonphosphorylated peptide under the same conditions did not. These results are in good agreement with those obtained by fluorescence quenching experiments, suggesting that the BIAcore provides a powerful technique for studying tyrosine phosphoprotein or phosphopeptide/SH2 interactions.

BZ 241 INTERACTION OF HUMAN C-*SRC* SH3- AND SH2-DOMAINS WITH CELLULAR PROTEINS IN HUMAN TUMOR-DERIVED CELL LINES. Deirdre K. Luttrell, Amanda Lee, Timothy J. Lansing, and Tona M. Gilmer, Dept. of Cell Biology, Glaxo Inc. Research Institute, Research Triangle Park, NC 27709.

Two distinct domains found in members of the *src* family of tyrosine kinases have been implicated in mediating protein-protein interactions. One of these, the src homology region 2 (SH2) domain, is a motif found in a number of proteins involved in intracellular signaling, including the non-catalytic subunit of phosphatidylinositol-3 kinase, phospholipase C-γ, the *ras* GTPase activating protein and both pp60 v- and c-*src*. The SH2 domain has been shown to mediate protein-protein interactions by binding to phosphorylated tyrosines on target proteins. The function of the src homology region 3 (SH3) domain is less well-understood. However, this motif is found in a number of proteins which associate with the cytoskeleton, such as myosin heavy chain, yeast actin binding protein and α-spectrin, and may serve to target proteins to certain regions of the cell. Little is known about the interactions of $pp60^{src}$ with other cellular proteins; however, mutations within either the SH2 or SH3 domain abrogate the transforming ability of $pp60^{v\text{-}src}$, and suggest that protein-protein interactions are likely to be critical to $pp60^{c\text{-}src}$ function as well. Since activation of the $pp60^{c\text{-}src}$ kinase has been reported in a number of human tumors, we are interested in examining the interactions of the SH2 and SH3 domains of the human c-*src* with target proteins in human tumor-derived cell lines. Using PCR, we have cloned the SH3 region (amino acids 87-143), SH2 region (amino acids 144-249) or the entire SH3:SH2 region (amino acids 87-249) of human c-*src* into the glutathione-S-transferase fusion protein vector pGEX-3X and expressed the fusion proteins in E. coli. The GST fusion proteins were collected on glutathione-sepharose from bacterial lysates, and have been used to affinity purify both tyrosine-phosphorylated and non-phosphorylated proteins from human colon and breast carcinoma cell lines. These proteins are currently being identified to determine relevant interactions between the SH3 and SH2 domains of human c-*src* with target proteins in human malignancies.

BZ 242 pp55, A NOVEL GASTRIC MUCOSAL TYROSINE KINASE: ROLE IN CELL PROLIFERATION, Adhip Majumdar, VA Medical Center, Allen Park, MI 48101 and Department of Medicine, Wayne State University, Detroit, MI 48201.

Tyrosine kinases (Tyr-k), which are associated with receptors of a number of growth factors and products of many proto-oncogenes, are thought to play an important role in the regulation of cell proliferation, differentiation and transformation. Since mucosal cells of the gastrointestinal tissues undergo constant renewal, we have been investigating the role of Tyr-k in regulating this process. We have demonstrated that stimulation of gastric mucosal proliferative activity whether the result of aging, injury or administration of EGF or bombesin to young animals, is associated with increased Tyr-k activity and tyrosine-specific phosphorylation of a membrane protein with M_r of 55 kDa (referred to as pp55). We postulated that pp55 may play a role in regulating gastric mucosal cell proliferation. In the initial characterization of pp55, we have raised polyclonal antibody to this protein (pp55- antisera). Subsequent immunoprecipitation of pp55 and affinity purified material showed that the pp55 in gastric mucosal membranes was a tyrosine kinase. Moreover, the gastric mucosa was found to have the highest pp55-associated Tyr-k activity when compared with the small intestine, colon, liver and brain. In addition, experimentally induced gastric mucosal cell proliferation also resulted in a rise in Tyr-k activity of pp55. Utilizing the pp55-antisera and a cDNA expression library derived from rat liver cDNA cloned into Lambda ZAP-II vector (Stratgene), we have isolated a candidate clone which cross reacted with pp55-antisera but not with antibody to $pp60^{c\text{-}src}$.

Supported by VA Med. Res. and NIH (AG 08438)

BZ 243 CHARACTERIZATION OF THE *TEC* PROTEIN-TYROSINE KINASE IN THE MURINE HEMATOPOIETIC SYSTEM, Hiroyuki Mano, Ken Sato, Yoshio Yazaki, James, N. Ihle* and Hisamaru Hirai, The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, JAPAN, *St. Jude Children's Research Hospital, Memphis, TN 38101-0813

Cytoplasmic protein-tyrosine kinases are presumed to be involved in mitogenic signaling pathway. However, it is still to be revealed which protein-tyrosine kinases play a key role in the mitogenic mechanism of myeloid cells. The *tec* kinase was recently identified as a novel member of non-receptor type protein-tyrosine kinases, and was shown to be preferentially expressed in liver.

Interestingly, we could reveal abundant expression of *tec* in a wide range of murine hematopoietic cell lines. The expression level of *tec* in these cells was no less than that of any cytoplasmic protein-tyrosine kinase which is known to be highly expressed in myeloid system.

Characterization of *tec* cDNAs in these cells further indicated that there are multiple forms of *tec* cDNAs generated by the mechanism of alternative splicing as follows. (1) An insertion of 41 bp in 5'-region of the cDNA results in replacement of N-terminus of the predicted *tec* protein. (2) An in-frame insertion of 66 bp into the region of SH3 domain introduces an additional 22 amino acids. (3) There is another insertion at 3'-region of the cDNA, replacing C-terminus of the protein. None of the forms above have a myristylation signal or a C-terminal tyrosine residue corresponding to Tyr 527 of the c-*src* protein. Detailed analysis of these alternative splicing by using RNA-dependent PCR will be presented.

It is currently under investigation to get insights of the *in vivo* role of the *tec* kinase.

BZ 244 MAPPING BIOLOGIC AND BICHEMICAL PROPERTIES

OF THE HUMAN fgr AND fyn GENES, Brona Matoskova,Jeanne H. Sameshima, Oliver Sartor, Keith Robbins,Laboratory of Cellular Development and Oncology,National INstitute of Dental Research Bethesda,*MD 20892*

We have observed that the oncogenic potential of the fyn proto-oncogene is approximately 10-fold greater than that of the fgr protooncogene. Furthermore ,upon activation, these gene products physically interact with a distinct set of substrates in fibroblasts. To map the genetic entities responsible for these biologic and biochemical properties, we have constructed recombinants between fgr and fyn genes.Reciprocal chimera containing heterologous unique, src-homology 2(SH2) and src-homology 3 (SH3) domains have been tested for their transforming activity by transfection. Using colony forming activity as a measure of transfection efficiency, preliminary data suggest that chimera containing the fgr kinase domain posses significantly higher transforming activity when compared to the normal fyn or fgr genes. Moreover, reciprocal chimera containing the fyn kinase domain lack detectable focus forming activity. Expression of each chimeric gene product has been verified by immunoblottig and the abundance of each protein per cell appears to be roughly equivalent. Currently, other biochemical parameters are being measured. These chimeric molecules provide us with a unique opportunity to identify substrates important for malignant transformarion in our fibroblast model system.

BZ 245 EN BLOC SUBSTITUTION OF THE SH2 DOMAIN ACTIVATES THE TRANSFORMING POTENTIAL OF THE c-Abl PROTEIN TYROSINE KINASE, Alexander J. Muller[1], Ann-Marie Pendergast[1], Kalindi Parmar[2], Marie H. Havlik[3], Naomi Rosenberg[2,4] & Owen N. Witte[1,3], [1]Molecular Biology Institute, Department of Microbiology and Molecular Genetics, and [3]Howard Hughes Medical Institute, University of California, Los Angeles, California 90024, Departments of [2]Pathology and [4]Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

Non-receptor protein tyrosine kinases share a common amino acid sequence motif with several other proteins involved in signal trasduction pathways termed the SH2 (*src* homology region 2) domain. In non-receptor protein tyrosine kinases, the SH2 domain has been implicated in influencing kinase activity and in mediating interactions with other proteins. Different SH2 domains exhibit distinct binding specificities for both phosphotyrosine- and phosphoserine/phosphothreonine-containing proteins. We have individually substituted sequences encoding the N-terminal SH2 domain of GAP (Ras GTPase activating protein) and the SH2 domain of Arg (*abl* related gene) en bloc into the c-*abl* proto-oncogene to assess the importance of native SH2 domain sequences within the context of c-Abl. SH2-substituted forms of c-Abl induced the transformed outgrowth of fibroblast colonies in agar, caused increased tyrosine phosphorylation of a predominant 62 kD cellular substrate, and localized to the cytoplasm rather than to the nucleus. These effects were more pronounced with the N-terminal SH2 domain of GAP than with the Arg SH2 domain. SH2 domain substitutions and the deletion of another region termed the SH3 domain had effects which were remarkably similar. Thus, these two domains may have coordinate roles as regulatory control elements in the context of c-Abl.

BZ 246 ferT- ENCODES A MEIOSIS SPECIFIC, NUCLEAR TYROSINE KINASE. **Uri Nir, Bella Hazan, Orna Bren, Miri Carmel and Flavio Lejbkowicz.** Department of Life Sciences, Bar-Ilan University, Ramat-Gan 52900, Israel

ferT is a mouse testis specific mRNA which accumulates solely in pachytene spermatocytes. These cells represent the longest stage in the first spermatogenic meiotic prophase. That pattern of expression is unprecedented by other tyrosine kinase encoding mRNAs, leaving ferT as the only meiosis specific **tyrosine kinase** encoding mRNA, described to date. ferT encodes a 51 KDa protein termed $p51^{ferT}$, which is the smallest tyrosine kinase described so far. By using anti $p51^{ferT}$ antibodies, we show that its accumulation pattern in the testes thus is restricted to the nucleus of cells residing in meiosis. Indirect immunofluorescense staining experiments, carried out in CHO cells expressing an exogenous ferT cDNA, under the control of a metallothionein IIA promoter confirmed the accumulation of $p51^{ferT}$ in the cell nucleus. The ectopic expression of ferT in mammalian cell lines, led to triploidy of these cells. This could be caused by different mechanisms. Amongst them are; a) interference of $p51^{ferT}$ with the mitotic chromosome segregation process; b) uncoupling of chromosome duplication and mitosis, by $p51^{ferT}$, in the transfected cells. A similar uncoupling process should take place during meiosis, allowing a DNA replication independent reductive cell division which results in haploid gametic cell. $p51^{ferT}$ may thus represent a new class of tyrosine kinases which are meiosis specific, reside in the cell nucleus and may be involved in uncoupling DNA replication from cell division.

BZ 247 THREE DIMENSIONAL SOLUTION STRUCTURE OF THE SRC HOMOLOGY 2 DOMAIN OF C-ABL.

Michael Overduin, Carlos B. Rios, Bruce Mayer, David Baltimore and David Cowburn. The Rockefeller University, New York, NY 10021

Src Homology 2 (SH2) regions are recognition motifs found in many intracellular signal transducing proteins that bind tyrosyl-phosphorylated protein sequences. The solution structure of the Abl SH2 product, a protein of 109 residues and 12.1 kDa, has been determined by multidimensional nuclear magnetic resonance methods. A pair of antiparallel β sheets and a C-terminal α helix enclose a hydrophobic core. A putative ligand binding groove is formed on an exposed portion of one β sheet between an N-terminal α helix and a five residue loop. Three arginine residues lie within this groove, their sidechains capable of ligating a phospho-tyrosyl group. Sequence homology comparisons to other members of the SH2 domain family show higher conservation in the hydrophobic core and binding area, suggesting a conserved global fold and mode of ligand binding.

BZ 248 THE TYROSINE KINASE TYK IN THE SIGNALLING PATHWAY OF α and β INTERFERONS. Sandra Pellegrini, Giovanna Barbieri, Laura Velazquez, Gilles Uzé* and Marc Fellous. INSERM U 276, Institut Pasteur, Paris, France; *Institut de Recherches Scientifiques sur le Cancer, Villejuif, France.

Cellular mutants provide a unique genetic system for dissecting a signal transduction pathway. The human mutant cell line 11,1 is insensitive to all interferon (IFN) α subtypes, weakly responsive to IFN β and normally responsive to IFN γ. The binding of labelled IFN α2 and α8 is impaired, while binding of IFN β takes place although with altered kinetics. Using a gene transfer approach we have identified and cloned the wild-type gene and the cDNA which restores binding and IFN sensitivity in the mutant cells. The gene encodes tyk (IFN-tyk), a known non-receptor tyrosine kinase with two tandem kinase domains. We have raised polyclonal antibodies recognizing IFN-tyk as a 130 kD protein associated with the membrane fraction of wild-type cells. IFN-tyk is phosphorylated on tyrosine residues shortly after treatment of cells with α and β IFNs. On the other hand, IFN γ has no effect on the amount or the phosphorylation state of the protein. Our genetic and biochemical analyses indicate that IFN-tyk is involved in the signalling pathways of α and β IFNs, but not of IFN γ. Binding studies assign a role for tyk in the formation of high affinity binding sites for α and β IFNs. Characterization of 11,1 transfectants expressing mutated forms of IFN-tyk will also be presented.

BZ 249 TWO-PHASE REGULATION OF THE CD4-p56^{lck} COMPLEX: ASSEMBLY AND AGGREGATION Monika Raab and Christopher E. Rudd Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney Street and the Department of Pathology, Harvard Medical School, Boston, MA 02115

p56^{lck} has been expressed in the baculoviral expression system in the presence of absence of its receptor CD4, and assessed by changes in enzymatic activity. The binding of *lck* was monitored by a comparison of kinase activity relative to the quantity of enzyme as detected by anti-lck immunoblotting. Enzyme was purified either by antibody affinity ,or DEAE chromatography, or directly assessed in whole cell lysates. Although several Mr forms of *lck* were expressed, an intermediate Mr form preferentially associated with CD4. Activity associated with the receptor bound form of the kinase was some 10-fold higher than that found in preparations of unbound kinase. These data indicate that the mere binding of p56^{lck} to CD4 is sufficient to activate the kinase and therefore indicate that the CD4-p56^{lck} complex is regulated differently from conventional tyrosine kinase receptors. The second stage of regulation was shown by the fact that antibody-mediated crosslinking further increased activity by 2 to 5 fold. We are presently investigating the molecular details of the mechanism of CD4-p56^{lck} regulation.

BZ 250 CHARACTERIZATION OF AN ABL SH3 BINDING PROTEIN **Ruibao Ren, Piera Cicchetti, Bruce Mayer and David Baltimore, Rockefeller University, New York, NY 10021**

SH3 (Src homology region 3) is a small protein domain containing about 50 amino acid residues. It is present in a very large group of proteins, including cytoskeletal elements and signaling proteins. In nonreceptor tyrosine kinases, deletion or mutation of the SH3 domain generally activates the transforming potential of proto-oncogene products, suggesting that SH3 mediates negative regulation of the kinase activity. A fusion protein containing glutathione-S-transferase fused to the SH3 domain of c-abl was used to probe a λgt11 cDNA expression library, and two clones that bound specifically to the SH3 were isolated. In this study, one of the clones, termed 3BP2, was characterized. 3BP2 is encoded by a single gene in mouse genome, which maps to chromosome 5 (Jenkins and Copeland, unpublished data). A 3.5 Kb 3BP2 transcript is expressed ubiquitously in mouse tissues at varying levels. Testis expresses an extra 2.8 Kb transcript. An antibody against 3BP2 recognizes proteins of 75, 70, 55 and 50 Kd in a variety of mouse and human cell lines. A 3Kb cDNA clone isolated from a λgt10 cDNA library contains an open reading frame encoding approximately 600 amino acid residues. In vitro transcription and translation of this cDNA gives rise to proteins predominantly at 80 and 75 Kd, which can be immunopreciptated by 3BP2 antibody. A comparative search of protein sequence databases has not been able to identify significant homologies to other known proteins. The associations of 3BP2 with c-abl and/or other proteins is being investigated.

The SH3 binding site in 3BP2 has been mapped to a 12 amino-acid region. A comparative search of protein sequence databases with SH3 binding sites of 3BP2 and 3BP1, another SH3 binding protein, reveals that some cellular and viral proteins contain sequences with homology to the SH3 binding motifs. The SH3 binding properties of these proteins is being examined.

BZ 251 HUMAN CD4+ CD45RA+ AND CD45RA- T CELL SUBSETS EXPRESS CATALYTICALLY ACTIVE CD4-p56^{lck} AND TcR/CD3-p59^{fyn} COMPLEXES AND POSSESS SIMILAR PATTERNS OF TYROSINE-KINASE SUBSTRATES. David M. Rothstein, Antonio da Silva, Kanji Sugita, Masahiro Yamamoto, Prasad Kanteti, Chikao Morimoto, Stuart F. Schlossman and Christopher E. Rudd. Division of Tumor Immunology Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115.

T cell activation is regulated by interacting protein tyrosine kinases (PTKs), and protein tyrosine phosphatases (PTP'ases). In man, the CD45 family of transmembrane PTP'ases is composed of five isoforms that are differentially distributed on subsets of T cells having distinct activation requirements and in vitro functions. The p56^{lck} and p59^{fyn} proto-oncogenes have been found to associate with CD4 and TcR/CD3, respectively. CD45 has been shown to play a critical role in T cell activation and is capable of regulating the activities of both of these receptor-associated PTKs in vitro. Given the potential regulatory interactions between CD45 and PTKs in CD4+ subsets expressing different CD45 isoforms, we examined CD45RA+ and CD45RA- CD4 peripheral blood T cell lines for differences in the presence of CD3-associated or CD4-associated PTK activity. In this study, we show that both subsets express CD4-associated p56^{lck} and TcR/CD3-associated p59^{fyn} kinases which exhibit identical in vitro phosphorylation at the Y-394 and Y-416 autophosphorylation sites, respectively. Both subsets also exhibited PI kinase activity associated with CD4-p56^{lck}. Consistent with these observations, anti-CD3 crosslinking induced the phosphorylation of an identical spectrum of intracellular substrates in these CD45RA+ and CD45RA- CD4+ T cell lines. These observations indicate that the mere expression of different CD45 isoforms does not in and of itself alter the presence or activity of receptor associated kinases or their intracellular targets.

BZ 252 CD5 ACTS AS A TYROSINE KINASE SUBSTRATE WITHIN A RECEPTOR COMPLEX COMPRISING TcRζ/CD3, p56^{lck} and p59^{fyn} Christopher E. Rudd, Masahiro Yamamoto, Kristine Burgess and K.V.S.Prasad Division of Tumor Immunology, Dana-Farber Cancer Institute, 44 Binney Street and the Department of Pathology, Harvard Medical School, Boston, MA 02115

CD5 is structurally related to the macrophage scavenger receptor family (SRCR), and can potentiate the proliferation of T-cells. In this study, we reveal a physical interaction between the CD5 antigen and the TcR/CD3 complex. Both anti-CD5 and anti-CD3 co-precipitated each other and the protein-tyrosine kinases p56^{lck} and p59^{fyn}. By contrast, anti-CD4 co-precipitated p56^{lck}, p32 and CD3/TcRζ subunits, but less CD5 suggesting the existence of CD4-TcRζ/CD3 complexes distinct from the CD5-TcRζ/CD3 complexes. Anti-CD5 mediated crosslinking induced tyrosine phosphorylation of numerous T-cell substrates, identical to those phosphorylated by TcRζ/CD3 ligation. Significantly, as in the case of the TcRζ chain, CD5 was found to act as a tyrosine kinase substrate induced by TcR/CD3 ligation. The kinetics of phosphorylation of CD5 ($T_{1/2}$=20sec) was amongst the earliest of activation events, more rapid than observed for theTcRζ chain ($T_{1/2}$=1min). CD5 represents a likely TcR/CD3 associated substrate for protein-tyrosine kinases (p56^{lck} or p59^{fyn}) involved in the generation of signals required for B-T collaboration.

BZ 253 ROLE OF SH2 AND SH3 DOMAINS OF *CSK* IN ITS ENZYMATIC ACTIVITY *IN VITRO* AND IN SUPPRESSION OF C-*SRC* KINASE ACTIVITY *IN VIVO*, Hisataka Sabe*, Akiko Hata*, Masato Okada#, Hachiro Nakagawa# and Hidesaburo Hanafusa*, *Laboratory of Molecular Oncology, The Rockefeller University, New York, NY 10021, #Division of Protein Metabolism, Institute for Protein Research, Osaka University, Suita, Osaka, 565, Japan

The regulation of c-Src kinase activity by phosphorylation of Tyr527 has been well characterized. CSK (C-terminal Src Kinase) has been identified as a kinase that specifically phosphorylates Tyr527 of c-Src *in vitro*. We have already demonstrated that CSK can suppress c-Src kinase activity *in vivo*. Although CSK has SH3 and SH2 domains in its N-terminal region, no stable complex formation between CSK and c-Src is detected. In an effort to explore how CSK recognizes and phosphorylates c-Src protein in vivo, we made a series of deletion or point mutations in the SH3 and/or SH2 domains of CSK. Deletion of almost the entire region of the SH3 and SH2 domains abolished CSK kinase activity and this mutant could not suppress c-Src kinase activity *in vivo*. Expression of the SH3 and SH2 domains of CSK could not suppress c-Src kinase activity. On the other hand, small deletions in the SH3 domain did not affect CSK kinase activity. However, these mutants could not suppress c-Src kinase activity *in vivo*. Deletion mutants of the SH2 domain could not accumulate within cells. Point mutants of the SH2 domain also abolished the suppression activities *in vivo* but not their enzymatic activities *in vitro*. These data suggest that in addition to the enzymatic activity, the SH3 and SH2 domains of CSK are necessary to suppress c-Src kinase activity *in vivo*. We have detected several CSK-binding proteins. The role of these CSK-binding proteins in regulating CSK kinase activity toward c-Src will be discussed. We shall also present data regarding the role of CSK in regulation of cell cycle-dependent oscillation of c-Src kinase activity.

BZ 254 VCP, THE MAMMALIAN HOMOLOG OF cdc48, IS TYROSINE PHOSPHORYLATED IN RESPONSE TO T CELL ANTIGEN RECEPTOR ACTIVATION, L. E. Samelson, M. Egerton, O. R. Ashe, D. Chen, B. J. Druker[1], and W. H. Burgess[2], Cell Biology and Metabolism Branch, National Institute for Child Health and Human Development, NIH, Bethesda MD 20892, [1]Division of Molecular and Cellular Biology, Dana Farber Cancer Institute, Harvard Medical School, Boston MA 02115, [2]Holland Laboratory, American Red Cross, Rockville MD 20850.

Activation of T cells through the T cell antigen receptor (TCR) results in the rapid tyrosine phosphorylation of a number of cellular proteins, one of the earliest being a 100kD protein. We have identified this 100kD substrate by partially purifying the protein using antiphosphotyrosine (APT) antibodies, amino acid sequence determination and cDNA cloning. We report here that the data show pp100 to be the murine equivalent of porcine valosin containing protein (VCP). Sequence analysis has shown VCP to be a member of a family of ATP binding, homo-oligomeric proteins, and the mammalian homolog of S. cerevisiae cdc48p, a protein essential to the completion of mitosis in yeast. We also provide proof that both endogenous and expressed murine VCP are tyrosine phosphorylated in response to T cell activation. Thus we have identified a novel component of the TCR mediated tyrosine kinase activation pathway that may provide a link between TCR ligation and cell cycle control.

BZ 255 **CLONING OF HUMAN *TEC* cDNAS AND ITS EXPRESSION IN HUMAN HEMOPOIETIC CELLS,** Ken Sato, Hiroyuki Mano, Yoshio Yazaki, and Hisamaru Hirai, The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Tokyo 113, JAPAN

Accumulating evidence has suggested that protein-tyrosine kinases are implicated in the mechanism of cell proliferation or transformation. Oncogenes encoding cytoplasmic protein-tyrosine kinases are actually known to be activated in many leukemic cells. The murine cytoplasmic protein-tyrosine kinase, *tec*, is expressed in a wide range of murine hemopoietic cell lines. Especially, the expression level of *tec* in myeloid cells is significantly higher than that of other cytoplasmic protein-tyrosine kinases, *jak*-1, *lyn*, or *hck* (as shown by Mano *et al* in this Symposium). These facts may suggest that *tec* plays a key role in the mitogenic signaling in hemopoietic system.

By using the murine *tec* cDNA as a probe, we could also reveal that *tec* is very abundantly expressed in the human hemopoietic cells including myeloid, B- and T-cell lineage. The *tec* kinase is, therefore, presumed to be a *key kinase* in human leukemic cells. To further analyze the function of human *tec*, we have tried to obtain human *tec* cDNAs from a T-cell line. Nucleotide sequencing of non-catalytic region of the human *tec* cDNAs demonstrates that the *tec* gene is highly conserved between the human and the mouse system (approximately 90 %). Determination of the primary structure of a full-length human *tec* cDNA and its expression among fresh leukemia samples is currently under investigation.

BZ 256 **STRUCTURAL AND FUNCTIONAL ROLE OF FYN IN T CELL RECEPTOR SIGNALLING.** Andrey S. Shaw, Lisa Gauen, Michael Olszowy, Lawrence E. Samelson, A. N. Tony Kong, Department of Pathology, Washington University, St. Louis, MO 63110, and CBMB, NIHCD, National Institutes of Health, Bethesda, MD 20892

Several lines of evidence link the protein tyrosine kinase, p59*fyn*, to the T cell receptor. The molecular basis of this interaction has not been established. Here we show that the tyrosine kinase, p59*fyn*, can associate with chimeric proteins that contain the cytoplasmic domain of CD3 ε, γ, ζ, and η. Mutational analysis of the ζ cytoplasmic domain demonstrated that the membrane proxiaml 41 residues of ζ are sufficient for p59*fyn* binding and that at least two p59*fyn* binding domains are present. The association of p59*fyn* with the ζ chain was specific as two closely-related src-family protein tyrosine kinases, p60*src* and p56*lck* did not associate with a chimeric protein that contained the cytoplasmic domain of ζ. Mutational analysis of p59*fyn* revealed that a ten amino acid sequence in the unique amino-terminal domain was responsible for the association with ζ. These findings support evidence that p59*fyn* is functionally and structurally linked to the T cell receptor. To test the function of p59*fyn* and identify critical signalling domains of p59*fyn*, we have overexpressed p59*fyn* in a T cell hybridoma and a cytotoxic T cell line. Overexpression of p59*fyn* strongly enhanced levels of secretion of IL2 after T cell activation supporting previous data suggesting that p59*fyn* is a rate-limiting factor in T cell activation. It had, however, no effect on cytotoxic T cell killing. This was a specific effect as overexpression of p60*src* had no effect on IL2 secretion or cytotoxicity. Surprisingly, overexpression of p56*lck* resulted in inappropriate IL2 secretion and enhanced cytotoxicity. Based on this data, we favor direct and distinct roles for p59*fyn* and p56*lck* in T cell activation.

BZ 257 THE ROLE OF TYROSINE PHOSPHORYLATION IN THE ACTIVATION OF THE NADPH OXIDASE OF HUMAN NEUTROPHILS. C.G. Teahan and A.W. Segal, Department of Medicine, The Rayne Institute, University College London, University St., London WC1E 6JJ, U.K.

The NADPH oxidase of neutrophils is a multicomponent system which transfers electrons from NADPH to molecular oxygen to generate toxic oxygen derivatives used in bacterial killing by these cells. An increase in tyrosine phosphorylation has been reported to accompany stimulation of the system but its relevance is unknown. We set out to investigate the role of tyrosine phosphorylation in regulation of the oxidase.

Unstimulated cells exhibit negligible levels of tyrosine phosphorylated proteins. Stimulation with a variety of agents give very similar patterns of tyrosine phosphorylation as detected by Western blotting using anti phosphotyrosine antibodies. In cells from patients with a disease known as chronic granulomatous disease (CGD), which fail to produce superoxide, there is no increase in tyrosine phosphorylation upon stimulation. This could be due to the fact that particular steps in the activation pathways are not being switched on or to the fact that the end products of the oxidase activity; superoxide, hydrogen peroxide etc, are not around to have any feedback effects on the cells.

We found that there is no increase in tyrosine phosphorylation when normal cells are stimulated under anaerobic conditions or in the presence of an inhibitor of the oxidase, diphenylene iodonium (DPI). The end products of the reaction, i.e. superoxide and hydrogen peroxide were tested for their ability to stimulate this phosphorylation and were found to lack any such effect. Thus tyrosine phosphorylation seems to be specifically associated with the activation mechanisms of the NADPH oxidase.

BZ 258 **DOES THE TYROSINE KINASE SUBSTRATE ANNEXIN II HAVE A ROLE IN EXOCYTOSIS FROM RAT BASOPHILS?** A. Louise Upton, Bastien Gomperts and Steven E. Moss, Department of Physiology, University College London, Gower Street, London, WC1E 6BT, UK.

The annexins are a family of calcium-dependent, phospholipid-binding proteins. They are conserved through evolution from humans to Dictystelium, Hydra and higher plants. Thirteen members of the family have been characterised so far. Several of them are known to be phosphorylated in whole cells. Annexin II (anx II) is the major cellular substrate for the transforming tyrosine kinase pp60$^{v\text{-}src}$ and anx I is the major cellular substrate for the EGF-receptor. Two important observations have implicated anx II in exocytosis: anx II can partially reverse the run down in secretory response of permeabilised adrenal chromaffin cells, and it can promote aggregation of chromaffin granules at physiological calcium concentrations. In an attempt to examine the proposed secretory role of anx II in intact cells we have adopted an approach employing transfection of a reverse orientation cDNA. To this end we have isolated and fully sequenced rat anx II clones from a plasmid library. We are presently attempting to stably transfect a rat basophilic cell line (RBL-2H3) with a vector containing antisense anx II cDNA. In this way we hope to "switch off" or reduce the levels of anx II and subsequently analyse the effect on secretory behaviour.

BZ 259 Crystal Structure of the Phosphotyrosine Recognition Domain (SH2) of the v-*src* Tyrosine Kinase Complexed With Tyrosine Phosphorylated Peptides, Gabriel Waksman†, Dorothea Kominos†, Scott C. Robertson†,D, John Kuriyan†,D,DHoward Hughes Medical Institute and †The Rockefeller University 1230 York Avenue, New York, NY 10021

Three-dimensional structures of complexes of the SH2 domain of the v-*src* oncogene product with two phosphotyrosyl peptides have been determined by X-ray crystallography at resolutions of 1.5 and 2.0 Å, respectively. A central antiparallel beta-sheet in the structure is flanked by two alpha-helices, with peptide binding mediated by the sheet, intervening loops, and one of the helices. The specific recognition of phosphotyrosine involves amino-aromatic interactions between lysine and arginine sidechains and the ring system in addition to hydrogen bonding interactions with the phosphate.

BZ 260 EXPRESSION, ISOLATION, AND CHARACTERIZATION OF AN N-TERMINAL FRAGMENT OF p56*lck*,
Cheryl L.Wilder[1], Andrey S. Shaw[2], Larry C. James[1], Department of Molecular Genetics and Protein Chemistry, Central Research Division, Pfizer Inc.,Groton, CT 06340[1], Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110[2]

p56*lck* is a *src*-related tyrosine kinase that participates in transducing antigen-induced signals in T lymphocytes. Like other *src*-related enzymes, p56*lck* consists of a unique N-terminal domain, two *src*-homologous domains (SH3 and SH2), and a C-terminal kinase domain. To study structure-function relationships in the N-terminal and *src*-related domains, we have expressed a mutant form of the enzyme which lacks the kinase domain. A gene encoding residues 1-228 of p56*lck*, with the additional sequence MMHHHHHHGS engineered at the C-terminus, was cloned into the T7 polymerase expression vector pET11d, and the resulting plasmid was expressed in *E. coli* strain BL21 (DE3). Expression of the recombinant protein was induced with IPTG and the cells were harvested 3 h later. p56*lck*(1-228) was isolated from the soluble fraction of cell lysates by anion exchange, gel filtration and metal affinity chromatography. The purified protein was characterized by N-terminal sequence analysis, mass spectrometry, IEF and SDS-PAGE.

BZ 261 PHOSPHORYLATION BY PKC AND MAP KINASE IN THE UNIQUE N-TERMINAL REGION OF THE TYROSINE KINASE P56LCK, David G. Winkler, Indal Park, Nicola S.Payne, Christopher T. Walsh, Jack L. Strominger, and Jaekyoon Shin, Division of Tumor Virology, Dana Farber Cancer Institute and the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115

The T cell specific tyrosine kinase, p56lck, is phosphorylated within the unique N-terminal (the first sixty six residues) region by Ser/Thr kinase(s) and dissociates from CD4 upon PMA treatment. Site directed mutagenesis and proteolytic mapping indicate that serine 42 and serine 59 are the major PMA induced phosphorylation sites. Phosphorylation of Ser 59 results in the observed gel-shift from 56 to 61 kD. Simultaneous phosphorylation of Ser 42 and Ser 59 results in a species migrating at 63 kD. In vitro kinase assays using peptides made from the Ser42 (**DGKGTLLIRNGSEVR**) and Ser59 (**DPLVTYEGSNPPASPLQ**) phosphorylation sites indicate that Ser42 can be phosphorylated by PKC and that Ser59 can be phosphorylated by MAP2-kinase. These results are consistent with a regulatory function for the N-terminal region of lck.

BZ 262 ALTERED TYROSINE KINASE ACTIVITY OF c-Src AND c-Yes DURING CALCIUM-INDUCED KERATINOCYTE DIFFERENTIATION. Yuhang Zhao, Hidesaburo Hanafusa, Marius Sudol, and James Krueger, The Rockefeller University, 1230 York Avenue, New York, NY 10021

Cultured human keratinocytes, like basal keratinocytes, maintain the ability to differentiate. When we induced differentiation in cultured keratinocytes by increasing intracellular calcium levels, the kinase activity of c-Yes was greatly reduced in a time dependent manner. In contrast, the c-Src kinase was activated in the same cells, despite high similarity to c-Yes in both protein structure and enzymatic activity. The protein levels of these two kinases during calcium treatment were unchanged, and both c-Src and c-Yes were dephosphorylated on tyrosine after calcium treatment. It is well established that dephosphorylation of the C-terminal tyrosine, as a negative regulatory mechanism, activates the activity of Src family kinases. Thus, the tyrosine dephosphorylation of c-Src in calcium-treated cells may explain our observed activation of this kinase, whereas the inactivation of c-Yes requires additional mechanism. We therefore investigated the possible association of potential regulatory proteins with c-Yes in calcium-treated cells, and found that c-Yes is indeed complexed with different cellular proteins after calcium treatment. Therefore, c-Yes kinase activity seems to be regulated by association with cellular proteins and this regulation may be strong enough to override the activation of its intrinsic kinase activity by dephosphorylation.

During the process of calcium-induced differentiation, there was a marked increase in nuclear phosphotyrosine content along with a translocation of both c-Yes and c-Src to the nucleus, as judged by immunofluorenscence microscopy with antibodies specific to phosphotyrosine or these two kinases. Thus, the homologous tyrosine kinases c-Yes and c-Src are regulated differently during keratinocyte differentiation, which is accompanied by changes in cellular tyrosine phosphorylation and their subcellular locations.

Receptor Serine/Threonine Protein Kinases

BZ 300 TGF-ß SIGNALS THROUGH A HETEROMERIC RECEPTOR COMPLEX, Liliana Attisano, Jeffrey L. Wrana and Joan Massagué, Howard Hughes Medical Institute and Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Centre, New York City, New York, 10021.

Transforming growth factor-ß, a member of the TGF-ß superfamily of cytokines potently inhibits the growth of Mink lung epithelial cells. This characteristic response was previously utilized to generate mink cell mutants which were non-responsive to TGF-ß and thus continued to proliferate in its presence. All other known responses to TGF-ß including extracellular matrix protein production were blocked. Characterization of several of these cell lines revealed the absence of both type I and type II receptors in DR cells and the absence of type I receptors in R cells. These cell lines were stably transfected with the recently cloned TGF-ß type II receptor and cellular responses to TGF-ß were measured. In the double-receptor (DR) mutants TGF-ß-induced responses were all restored. In contrast, R mutants, lacking the type I receptor did not recover any TGF-ß responses. Furthermore, transfection of the type II receptor alone into DR cells, resulted in the expression of type I receptor on the cell surface. Co-immunoprecipitation of the type I receptor with the type II receptor shows that these two proteins form a complex on the cell surface. These findings demonstrate that receptors I and II associate as interdependent components of a heteromeric complex: receptor I requires receptor II to bind TGF-ß and receptor II requires receptor I to signal.

BZ 301 DEVELOPMENT AND APPLICATIONS OF A SYNTHETIC TGF-β -RESPONSIVE PROMOTER.

Juan Carcamo and Joan Massague. Cell Biology and Genetics Program and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

We developed a TGF-β-responsive luciferase reporter gene (p3TP-Lux). Of various TGF-β-responsive promoters constructed, the 3TP promoter which contains three consecutive TRE elements and a portion of the plasminogen activator inhibitor-1 (PAI-1) promoter region was found to mediate the highest inducibility by TGF-β. The 3TP promoter is inducible by TGF-β in a variety of mammalian cell lines.

Using this assay system we have screened a panel of TGF-β resistant cell mutants that express TGF-β receptors I and II but fail to respond to TGF-β. Luciferase activity was determined in extracts from cells transiently transfected with p3TP-Lux alone or cotransfected with human TGF-β receptor II (hTβR-II). All except two of these cell lines showed no responsiveness to TGF-β after transfection of hTβR-II. The two cell lines whose responsiveness to TGF-β was rescued by hTβR-II are likely to contain mutations that disrupt the signaling activity of the endogenous receptor II. Cloning and sequencing of this receptor are underway to determine the nature of these mutations.

BZ 302 A SERINE/THREONINE SPECIFIC PROTEIN RECEPTOR KINASE IS INVOLVED IN THE SELF-INCOMPATIBILITY RESPONSE IN Brassica napus. Tracy L. Glavin, Daphne R. Goring and Steven J. Rothstein. Department of Molecular Biology and Genetics, University of Guelph, Guelph, Canada N1G 2W1

Self-incompatibility (SI) is one mechanism that prevents inbreeding in flowering plants. In self-incompatible plants, the germination of self-pollen is prevented by the interaction of pollen and stigma cells. In Brassica, the SI response is controlled by a single, multi-allelic locus, the S-locus. Pollen germination is inhibited when pollen and stigma papillae cells from plants carrying the same S-locus alleles interact. Two known genes exist at this locus. The first gene (S-locus glycoprotein (SLG) gene) codes for a secreted glycoprotein, while the second gene (S-receptor kinase (SRK) gene) encodes a receptor protein kinase. We are studying the SLG and SRK genes in self-incompatible Brassica napus ssp oleifera lines produced by the introgression of S-alleles from SI B. campestris and SI B. napus ssp rapifera plants into self-compatible B. napus plants. We have isolated SLG and SRK cDNAs from two SI B. napus lines and have shown that the SRK protein is a serine/threonine kinase. In segregating F2 populations, these SLG and SRK genes segregate with the SI phenotype. We have also isolated a set of these genes that do not segregate with the SI phenotype in F2 populations. In this case, the SRK gene contains a deletion that would produce a frame-shift mutation. This mutation may account for the inability of this SLG/SRK gene pair to produce a SI response. We are attempting to identify which regions of the extracelluar domain of the SRK are important for ligand interactions and subsequent induction of kinase activity.

BZ 303 BETAGLYCAN INTERACTS WITH THE TGF-ß SIGNALING RECEPTOR, Fernando López-Casillas, Jeffrey L. Wrana and Joan Massagué, Howard Hughes Medical Institute and Memorial Sloan-Kettering Cancer Center, New York, NY 10021.

Betaglycan (BG, also known as the type III TGF-ß receptor) is a cell surface proteoglycan that binds TGF-ß and coexists in many cell types with the signaling TGF-ß receptors (the type I and type II receptors). Direct evidence for a TGF-ß signaling function for betaglycan has not been found and its cytoplasmic tail does not exhibit any signaling motif. However, we report that BG forms a complex with the type II TGF-ß receptor, a transmembrane serine-threonine kinase that mediates TGF-ß action. This complex can be demonstrated by immunoprecipitations with antibodies directed against either BG or the type II receptor. This association is dependent on the presence of the ligand and is non-covalent in nature. We have detected this complex in a variety of cell lines.

When BG is transfected into L6E9 myoblasts that normally do not express BG, TGF-ß binding to the type II receptor is enhanced. In addition, these cells become more sensitive to long term TGF-ß responses as compared to the parental cell line.

These results suggests a role for BG as an accessory molecule that may present the ligand to the TGF-ß receptors through a direct interaction with this receptor, thereby increasing the efficacy of the cell interaction with TGF-ß.

BZ 304 STUDIES ON THE COMMON PHYLOGENETIC ORIGINS OF TRANSMEMBRANE KINASE RECEPTORS. Andrew Sizeland, Sean E. Egan, Feng Chen and Robert A. Weinberg. Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142.

Several receptors for transforming growth factor ß have been identified by cross linking iodinated TGFß to various cells. The type II receptor has recently been cloned in this laboratory and found to be a serine/threonine kinase and a member of a large family of structurally related transmembrane protein kinases. It is possible that both serine/threonine and tyrosine kinase cell surface receptors arose evolutionarily from the same common precursor, but functional evidence of this is lacking. We have prepared chimeras of the EGF and TGFß receptors and expressed these in cells lacking the parental receptor (either the TGFß or EGF receptors). The biological responses of the cells to the appropriate ligands will then be examined and compared to the responses elicited in cells expressing the parental type II TGFß receptor or the EGF receptor.

BZ 305 MOLECULAR CLONING OF NOVEL HUMAN PROTEIN SERINE/THREONINE KINASE RECEPTORS, Peter ten Dijke, Hidenori Ichijo, Petra Franzén, Peter Schultz, Carl-Henrik Heldin and Kohei Miyazono, Ludwig Institute for Cancer Research, Uppsala branch, Box 595, S-751 24 Uppsala, Sweden

The transforming growth factor-beta (TGF-beta) superfamily consists of a family of structurally related proteins, which include TGF-betas, activins, inhibins, Mullerian inhibiting substance and bone morphogenetic proteins. Recently, the mouse activin receptor II (mActRII) cDNA was cloned (Mathews and Vale 1991, Cell 65, 973-982) and found to possess sequence homology with the C. elegans daf-1 gene (Georgi et al. 1990, Cell 61, 635-645). The intracellular domain of mActRII contains a domain which predicts a serine/threonine (S/T) specific protein kinase activity. Predicting that receptors for the TGF-beta superfamily members may possess sequence similarity to each other, we designed a polymerase chain reaction (PCR) strategy with degenerate primers based upon the amino acid sequence similarity between the mActRII and daf-1 gene products. PCR products were obtained, which were derived from ActRII, ActRIIB (Attisano et al, 1992, Cell 68, 97-108) and TGF-betaRII (Lin et al, 1992, Cell 68, 1-21). In addition, several PCR products were derived from novel genes, which showed a 30-40 % sequence similarity with ActRII and TGF-betaRII and a 60-70 % sequence similarity towards each other in the S/T kinase domain. Probing Northern blots with PCR products showed different expression patterns for the different genes. Human cDNA clones for some of the genes have been obtained.

BZ 306 *RLK5*: A FUNCTIONAL SERINE/THREONINE RECEPTOR PROTEIN KINASE FROM *ARABIDOPSIS THALIANA*, John C. Walker and Mark A. Horn, Division of Biological Sciences, University of Missouri, Columbia, MO 65211

Molecular cloning of protein kinase genes from higher plants has recently led to the discovery of a novel family of serine/threonine protein kinase receptors. These were first discovered in maize but subsequent studies have found members of this family in *Brassica* and *Arabidopsis*. To date two classes of receptor-like protein kinase (RLK) genes have been identified in plants. The Class I RLK genes have been identified in maize (*ZmPK*1 and *ZmPK*2), Arabidopsis (*RLK*1, *RLK*4, and *ARK*1), and *Brassica* (the alleles *SRK*2 and *SRK*6). The predicted extracellular (amino-terminal) domain of the Class I RLKs has 10 conserved cysteine residues clustered near the transmembrane domain and several potential N-linked glycosylation sites. This domain is related to the self-incompatibility locus glycoproteins (SLGs) of Brassica. For example, the prototypical Class I RLK, ZmPK1, is 27% identical and 52% similar to the *Brassica oleracea s13* SLG. The Class II RLK genes are characterized by having an extracellular domain that consists of several leucine rich-repeats with a unit length of 24 amino acids. A similar repeating motif has been found in several other proteins from humans, flies and yeast. This repeating structure is thought to interact with other macromolecules and may be important in mediating cell to cell, cell to protein, or protein to protein interactions. Thus far, the Class II RLK genes have been identified only in *Arabidopsis thaliana*: RLK5 has 21 leucine-rich tandem repeats, while the other Class II RLK gene has 11 leucine-rich tandem repeats.

We have expressed the catalytic domain of RLK5 in *E. coli* and have demonstrated that it autophosphorylates on both seryl and threonyl amino acids, but there is no apparent autophosphorylation on tyrosine. Autophosphorylation of the recombinant RLK5 protein kinase occurs by intramolecular mechanism. The Km is 60µM for ATP and the Vmax is approximately 120 pmol ATP/mg·min. Current studies are focusing on the identification of substrates for RLK5 kinase in plant tissues.

BZ 307 STRUCTURAL AND FUNCTIONAL ELEMENTS OF THE TGF-BETA RECEPTOR TYPE II: ROLE OF THE C-TERMINAL TAIL FOR SIGNALLING Rotraud Wieser, Jeffrey Wrana, and Joan Massague, Program of Cell Biology and Genetics, Memorial Sloan Kettering Cancer Center and Howard Hughes Medical Institute, New York, New York 10021.

The TGF-beta receptor type II is a transmembrane protein that shows significant homology to serine/threonine protein kinases, as do receptors for other factors of the TGF-beta superfamily. The TGF-beta type II receptor appears to be closely associated with the TGF-beta receptor type I, with both molecules being necessary to allow responses to TGF-beta to occur.

Like many transmembrane protein tyrosine kinases, the TGF-beta type II receptor is capable of autophosphorylation in vitro and is a highly phosphorylated molecule in intact cells. The region of the type II receptor that is located C-terminal of the kinase domain (the receptor tail) is rich in serine and threonine, residues that are potential targets for autophosphorylation. In analogy with transmembrane protein tyrosine kinases, autophosphorylated residues might be involved in substrate recognition. Therefore, the importance of this region for TGF-beta signalling was investigated. A truncated version of the type II receptor with a deletion of the C-terminal tail was introduced into mutants that otherwise lack a functional type II receptor. This deletion neither affects the interaction with the type I receptor, nor does it abolish the response of an artificial TGF-beta inducible promoter, as assessed in a transient transfection assay. Stable transfectants are available now to investigate other responses to TGF-beta. Nevertheless, the data so far available suggest that the serine-threonine-rich tail of the receptor is not required for the molecule to be functional.

BZ 308 ANALYSIS OF TRANSFORMING GROWTH FACTOR BETA-1 SIGNAL TRANSDUCTION IN RAT LIVER EPITHELIAL CELLS
Peter J. Wirth and Lin-di Luo, Laboratory of Experimental Carcinogenesis, National Cancer Institute, NIH, Bethesda, MD 20892.
Transforming growth factor beta (TGF-β) is a family of growth regulatory polypeptides that is a potent inhibitor of DNA synthesis and cellular proliferation of many cultured cells of epithelial origin including rat liver epithelial (RLE) cells. In order to gain insight concerning the mechanism of TGF-β1 mediated signal transduction we investigated the expression of silver stained, [^{35}S]-methionine- and [^{32}P]-orthophosphate labeled whole cell and nuclear polypeptides in RLE cells immediately after treatment with TGF-β1 (5 ng/ml) using high resolution 2D-PAGE. Although TGF-β1 had no significant effect on overall protein synthesis within 15 min of addition of TGF-β1 the phosphorylation of a 43 kDa and a number of high M$_r$ (116-120 kDa) nuclear polypeptides were significantly increased. Concomitant with these increases were decreases in the phosphorylation of polypeptides 2 (5.00/85 kDa) and 3 (4.90/84 kDa). The expression of polpeptides 2 and 3 returned to constitutive levels within 60 min. These effects were the result of increased phosphorylation and dephosphorylation, respectively, since no changes in the extent of [^{35}S]-methionine labeling of the corresponding polypeptides were observed. In synchronized RLE cell populations TGF-β1 induced a marked increase in the phosphorylation of a relatively acidic polypeptide (pI 6.90/22 kDa) within 15 min which returned to constitutive levels after 60 min. Phosphorylation of this polypeptide was limited to late G1 in the cell cycle. Our results indicate that TGF-β1 induces rapid and transient modulation of the phosphorylation status of specific subsets of polypeptides and these effects appear to be cell cycle dependent. It is conceivable that included among these are specific phosphoproteins that may be involved in the modulation of the growth inhibitory action of TGF-β1 on RLE cells.

BZ 309 TGF-ß RECEPTOR II KINASE ACTIVITY IS REQUIRED FOR SIGNALLING BY THE HETEROMERIC TGF-ß RECEPTOR COMPLEX. Jeffrey L. Wrana, Liliana Attisano, and Joan Massagué, Howard Hughes Medical Institute and Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Centre, New York City, New York, 10021.
Transforming growth factor-ß, a member of the TGF-ß superfamily of cytokines potently inhibits the growth of epithelial cells. This response has been utilized to generate a number of mink lung epithelial cell lines that display abnormal TGF-ß receptor profiles and lack TGF-ß responsiveness. R mutants are characterized by the absence of type I receptors while DR mutants lack both type I and type II. Stable expression of the TGF-ß type II receptor (TßR-II) in these cells rescues all TGF-ß responses in DR but not R mutants. Furthermore, expression of TßR-II in DR cells resulted in the appearance of type I receptors and immunoprecipitation analysis demonstrates that the two receptors form a complex. Together these results show that type I requires the presence of type II to interact with ligand and that type II requires type I for signalling. To investigate the functional requirements necessary for signalling by the receptor complex we have assessed the ability of mutated type II receptors to signal TGF-ß responses in DR cells. The highly conserved lysine, implicated in nucleotide binding by kinases, was converted to an arginine to create a kinase deficient receptor. This modified TßR-II when expressed in DR cells formed a complex with receptor I, however no TGF-ß responses were rescued indicating that a functional type II kinase domain is essential for signalling by the receptor complex. Currently we are extending our studies to investigate the function of the kinase insert regions in mediating signal transduction by the TGF-ß receptor.

BZ 310 CHARACTERIZATION OF MUTATIONS IN THE MINK TGF-ß TYPE-II RECEPTOR CONFERRING RESISTANCE TO GROWTH INHIBITION, Alejandro Zentella, Liliana Attisano and Joan Massague', Program of Cell Biology and Genetics, Memorial Sloan Kettering Cancer Center & Howard Hughes Medical Institute, New York , NY 10021

The type II receptor for TGF-ß (TßR-II) contains a ligand-binding domain in the extracellular region and a seryl-, threonyl-kinase domain in the intracellular portion of the molecule.
Through chemical mutagenesis several clones of mink lung epithelial cells (Mv1Lu) resistant to the growth inhibitory effect of TGF-ß were isolated, comprising mutations that lead to a defective ligand binding of both type I and type II receptors or double receptor mutants (DR) and mutants with normal ligand binding for both receptors but deficient in signaling (S) (Laiho, M. et al. JBC 265:18518-24 1990).
We have initiated the characterization of these cells by cloning and sequencing the mink TGF–ßRII from wild type and the two double receptor clones DR-26 and DR-27.
The predicted mink TßR-II amino acid sequence is overall 94% identical to the human TßR-II, with 77% and 97% conservation in the ligand binding and kinase domains respectively.
In DR-26 a (C->T) change introduced a stop codon in the seventh codon of the transmembrane region, predicting a truncated receptor that, if stable, is likely to be released into the medium.
In DR-27 a (G->T) change substituted the third cysteine of the extracellular domain for a tyrosine, suggesting that this cysteine residue is essential in maintaining a functional ligand binding domain.

Non-Receptor Serine/Threonine Protein Kinases

BZ 311 FURTHER CHARACTERIZATION OF AN ECTO-KINASE CAPABLE OF PHOSPHORYLATING BASIC FGF AT THE CELL SURFACE OF TARGET CELLS, P. Auguste, N. Boulle, R. Matsunami, E. Amburn, P. Maher, and A. Baird, Department of Molecular and Cellular Growth Biology, The Whittier Institute for Diabetes and Endocrinology, La Jolla, CA 92037

Although a phosphorylated form of basic fibroblast growth factor (bFGF) can be immunoprecipitated from the human hepatoma cell line SK-Hep, it is not clear which enzyme phosphorylates this growth factor (*PNAS 86*:3174-3178, 1989). Recently we have attributed a component of this activity to two novel enzymes present on the outer cell surface of target cells (*Mol. Endo. 5*:1003-1012, 1991). The first SK-Hep-derived ectokinase activity is partially activated by cAMP, but differs from the PK-A family of kinases in that the phosphorylation of bFGF is inhibited by heparin.

The second ectokinase present on the outer cell surface of SK-Hep which we are also interested in is non activated by cAMP. This ectokinase is activated by $MgCl_2$ and by physiological concentrations of $CaCl_2$ (2-3 mM). $MgCl_2$ could be substituted by micromolar concentrations of $MnCl_2$. The kinase activity is also modulated by heparin, suggesting that *in vivo* there may be an interaction between the ectokinase and heparin sulfate related glycosaminoglycans. The activity is widely distributed and is readily detectable on different FGF target cell types, including retina pigmented epithelial cells and fibroblasts.

The existence of a protein kinase that is both associated with the extracellular membrane of SK-Hep cells and capable of phosphorylating bFGF suggests this post-translational change may modulate bFGF activity. For this reason we investigated the possible role of this phosphorylation on signal transduction. To this end, we examined the effects of 5'-deoxy-5'-methyl-thioadenosine (DMTA), an inhibitor of methylase, that can inhibit the tyrosine kinase activity associated with the bFGF receptor, but does not inhibit the binding of bFGF to its receptor. We find that DMTA also inhibits the phosphorylation of bFGF in a dose dependent manner (IC_{50} 2 mM). Taken together, the results suggest the phosphorylation of bFGF by SK-Hep cells is mediated by a novel ectokinase which may be involved in the regulation of this growth factor's availability.

BZ 312 A MOLECULAR GENETIC ANALYSIS OF ERK KINASE FUNCTION DURING DEVELOPMENT IN *DROSOPHILA MELANOGASTER*, William H. Biggs, Helmut Krämer, and S. Lawrence Zipursky, HHMI, Dept. of Biological Chemistry, University of California, Los Angeles, Los Angeles, CA 90024-1662.

The Extracellular Signal-Regulated Protein Kinases (ERKs) comprise a class of protein-serine/threonine kinases that are activated in response to a wide variety of extracellular signals transduced via receptor tyrosine kinases. Activation of the ERKs requires both threonine and tyrosine phosphorylation suggestive of a key role in mediating intracellular events in response to extracellular cues. To critically assess the role of ERKs in intracellular signaling, a genetically tractable receptor tyrosine kinase system would be invaluable.

Through the use of RT-PCR we have identified a *Drosophila melanogaster* homolog of rat ERK1 and -2, designated DmERK-A. The deduced amino acid sequence of DmERK-A predicts a protein of ~43kD *which is 80% identical* to rat ERK1 and -2. An affinity purified antiserum raised against DmERK-A recognized a single band of ~44kD in a western analysis of tissue isolated from several stages of development. Similar analysis also indicated that DmERK-A is expressed in the *Drosophila* S2 embryonic cell line. We also demonstrated that DmERK-A expressed in the S2 cell line underwent a rapid and transient phosphorylation on tyrosine residues in response to insulin treatment. The biochemical and sequence similarity of DmERK-A to rat ERK1 and -2 have led us to propose that DmERK-A is a functional homolog of the vertebrate ERK kinases.

DmERK-A has been cytologically localized to band 45A2 on the second chromosome. We have recently obtained a cytologically visible deletion and several embryonic lethal mutations which have been mapped to this same region. These reagents are now being analyzed to determine if any represent loss-of-function mutations in DmERK-A. In addition we have generated S2 cell lines , as well as transgenic flies which express two potential dominant negative forms of DmERK-A. Preliminary experiments with these S2 cell lines indicated that catalytically inactive DmERK-A undergoes tyrosine hyperphosphorylation in the absence of insulin treatment. The implications of these results for ERK function will be discussed. Progress on the genetic analysis of DmERK-A will be presented

BZ 313 OPPOSING IMPACT OF TWO PKC ISOFORMS ON CELL GROWTH AND TRANSFORMATION, Borner, C., Ueffing, M., and Weinstein, I. B. Comprehensive Cancer Center, Columbia University, New York, NY 10032

Two closely related protein kinase C (PKC) isoforms, PKCβI and PKCα, were overexpressed in R6 embryo fibroblasts. This had an astonishingly divergent impact on growth and transformation of these cells. While PKCβI-cells grew faster, reached high saturation densities and were partially transformed, PKCα-cells were growth inhibited and did not acquire a transformed phenotype. To find an explanation for this difference, we investigated the impetus of the overexpressed PKCs on positive and negative regulatory components of cellular growth. Both, PKCβI- and PKCα-cells elicited a exaggerated and persistent change in morphology as well as a higher and sustained inducibility of immediated early and secondary genes in response to phorbol esters. Concomitantly, both cell derivatives exhibited enhanced negative transmodulation of EGF binding and enhanced inhibition of diacylglycerol production. Therefore, overexpression of PKCα and PKCβI appears not only to facilitate, but also to further restrict mitogenic signalling, consistent with the proposed dual action of PKC on growth. PKCβI-cells had, however, a unique feature; they acquired the capacity to overcome enhanced negative feedback regulation by releasing into the medium a putatively autocrine-acting growth factor which synergized with EGF and PDGF to stimulate DNA synthesis. This factor is not secreted by PKCα-cells and may therefore be the causative agent for the proliferative and transformed phenotype of PKCβI-cells.

BZ 314 Redundant pathways of ERK2 activation in EGF signaling. Boudewijn M.Th. Burgering; Alida M.M. de Vries-Smits; René H. Medema; Pascale C. van Weeren; Johannes L. Bos. Laboratory of Physiological Chemistry, Utrecht University, Vondellaan 24A, 3521 GG, Utrecht, The Netherlands.

Many growth factors activate both p21ras and members of the extracellular regulated kinases (ERKs). Recently, we[1] and others have shown that p21ras is essential for the activation of ERK2 by some of these growth factors. In this study we analyzed whether in fibroblasts, p21ras-independent signaling pathways contribute to the activation of ERK2 induced by EGF, PDGF, TPA, Ca^{++} and insulin. It was found that in case of insulin and PDGF, ERK2 activation appears entirely mediated by p21ras, in contrast ERK2 activation by TPA is not mediated by p21ras and requires activation of protein kinase C (PKC). The activation of ERK2 by EGF appears to be mediated by two different pathways. The first pathway involves activation of p21ras and the second is dependent on extra-cellular Ca^{++}. This latter conclusion is based on the observation that blocking either pathway prior to EGF stimulation does not inhibit ERK2 activation significantly, whereas combined inhibition of Ca^{++} influx and p21ras activation results in full inhibition of ERK2 activation. These results demonstrate that within one cell type at least three parallel pathways exist that can lead to activation of ERK2. These pathways can be differentially employed by growth factors but all pathways converge towards ERK2 activation.

1. de Vries-Smits et al. Nature **357**, 602-604 (1992).

BZ 315 CHK, A NOVEL MEMBER OF THE HELIX-LOOP-HELIX FAMILY OF PROTEINS THAT CONTAINS A SERINE-THREONINE KINASE DOMAIN, Margery A. Connelly and Kenneth B. Marcu, Departments of Pathology and Biochemistry and Cell Biology, SUNY at Stony Brook, Stony Brook, NY 11794-5215.

Oligonucleotide primers were made to two conserved areas of the murine *c-myc* gene's polypeptide: an acidic domain and an amphipathic helical domain known to be required for protein-protein interactions and specific DNA binding. A reverse transcriptase/polymerase chain reaction (RT-PCR) protocol was employed and one of the products was cloned revealing a novel helix-loop-helix (H-L-H) domain without an adjacent basic region. The nucleotide sequence of its full length cDNA clone revealed a putative polypeptide not only containing an H-L-H domain at its carboxy-terminus but more remarkably, a serine-threonine kinase catalytic domain at its amino-terminus. Therefore, this novel gene now called CHK, conserved helix-loop-helix kinase, may represent a new class of chimeric proteins. Northern blot analyses revealed that CHK is ubiquitously expressed as a 3.7 kb transcript and a cross-species Southern blot showed strong evolutionary conservation. Peptide specific polyclonal antisera are being used to identify its polypeptide products and potential *in vivo* functions.

BZ 316 STIMULATION OF pp44 and pp42 MITOGEN-ACTIVATED PROTEIN KINASES BY ANGIOTENSIN II IN CULTURED RAT AORTIC SMOOTH MUSCLE CELLS. Marshall A. Corson, Jennifer L. Duff and Bradford C. Berk, Division of Cardiology, Emory University, Atlanta, GA 30322

Vasoconstrictors such as angiotensin II stimulate vascular smooth muscle cell (VSMC) hypertrophy and share many signal transduction mechanisms with growth factors, including activation of phospholipase C, protein kinase C and Na^+/H^+ exchange. Recently many growth factors have been shown to activate mitogen-activated protein (MAP) kinases, a family of serine/threonine protein kinases which phosphorylate $pp90^{rsk}$, a cytosolic kinase that activates ribosomal S6 kinase, resulting in increased protein synthesis. To investigate whether vasoconstrictors also activate MAP kinases, we examined the effect of angiotensin II on $pp44^{mapk}$ and $pp42^{mapk}$ activation in cultured rat aortic VSMC. Angiotensin II (200 nM) stimulated the enzymatic activity of MAP kinase by 4-fold after 5 min of exposure; the EC_{50} for MAP kinase activity (5 nM) corresponded to that previously observed for angiotensin II-induced VSMC hypertrophy. Angiotensin II also stimulated tyrosine phosphorylation of 42 kDa (73 ± 42% increase at 5 min) and 44 kDa (263 ± 95%) proteins. These proteins were shown to be $pp42^{mapk}$ and $pp44^{mapk}$ by Western blot analysis using an antibody generated in response to AA 325-345 of $pp42^{mapk}$. These findings demonstrate another signal transduction mechanism in VSMC shared by growth factors and vasoconstrictors. Because MAP kinases phosphorylate and activate $pp90^{rsk}$, these results suggest that angiotensin II-stimulated protein synthesis, and hence hypertrophy, is mediated by a MAP kinase dependent pathway.

BZ 317 PROTEIN KINASE C IN RAT LIVER NUCLEI. Mariëlle P. De Moel, Sjenet E. Van Emst-De Vries, Peter H.G.M. Willems and Jan Joep H.H.M. De Pont, Department of Biochemistry, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

More and more elements of the phosphatidylinositol/calcium signal transduction pathway, originally thought to be present only in plasma membrane, endoplasmic reticulum and cytosol, are recently demonstrated to be also localized in the nucleus or nuclear membranes. One of these elements is protein kinase C (PKC) and because of its potential role in the nucleus we started a study on PKC in rat liver nuclei. We investigated presence, subtype identity and localization of this enzyme. In addition, identification of endogenous nuclear substrates is carried out. This is important because knowledge of the intranuclear localization of PKC and its substrates will help to elucidate nuclear function and organization.

Nuclei were purified from rat liver essentially as described by Blobel and Potter ((1966) Science, 154, 1662-66). Proteins were solubilized from nuclei by Triton X-100 extraction and sonication. The 100,000 x g supernatant was defined as nuclear extract. Both in a suspension of nuclei and in the nuclear extract we demonstrated Ca^{2+}, phosphatidylserine and phorbol ester stimulated phosphorylation. Optimum assay conditions were determined for the nuclear extract. PKC was further purified by DEAE-Sepharose chromatography. In addition PKC isotypes were analyzed in intact nuclei, nuclear extract and purified fractions with isotype-specific polyclonal antibodies (anti-PKC α, β, γ, δ, ϵ and ζ) using immunocytochemistry and western blotting.

BZ 318 Development of antisense oligonucleotides targeting human PKC-α. **Nicholas M. Dean, Robert McKay and C. Frank Bennett. Isis Pharmaceuticals, 2280 Faraday Ave., Carlsbad CA 92008**

Protein kinase C (PKC) consists of a family of protein serine/threonine kinases which can be sub-divided into two major groups, those with Ca^{2+} binding domains (C2) (PKC-α,βI,βII and γ), and those without (PKC-δ,ε,ζ and η). Levels of PKC-α are reversibly lowered by prolonged treatment of human lung carcinoma (A549) cells with phorbol 12, 13 dibutyrate (PDBu) and this response has been used to determine the ability of various oligonucleotides to inhibit resynthesis of PKC-α protein subsequent to PDBu withdraw. A series of 20 mer phosphorothioate oligonucleotides complementary to the 5'-untranslated region, the AUG translation initiation codon, internal domains and the 3' untranslated region of human PKC-α mRNA have been examined. We have found that some oligos from each of these regions are capable of inhibiting PKC-α protein synthesis, however those targeting regions in the 3'UTR were the most active. In the presence of cationic lipids, our most potent oligonucleotides exhibit IC_{50} values of approximately 100nM. In addition, all of our active phosphorothioate oligonucleotides lower PKC-α mRNA. This was suggestive that RNAse H mediated cleavage of oligonucleotide targeted mRNA was occuring. To support this hypothesis we examined the ability of 2'-O-methyl/phosphorothioate analogs of our active oligos to lower PKC-α mRNA. This chemical modification increases both nuclease resistance and hybridization affinity, but does not support RNAse H mediated hydrolysis of any hybridizing RNA. We found that oligonucleotides with this chemical modification were unable to decrease PKC-α mRNA and were generally without effect on PKC-α protein synthesis. The exception to this was an oligonucleotide targeting the AUG initiation of protein synthesis codon which was able to inhibit PKC-α protein synthesis. This suggests that although RNAse H mediated hydrolysis of mRNA increases oligonucleotide potency, it is not absolutely required for oligonucleotide activity.

BZ 319 A NOVEL, REGULATED NUCLEAR KINASE IN MAMMALIAN CELLS. Gerald V. Denis and Michael R. Green, Program in Molecular Medicine, University of Massachusetts Medical Center, Worcester, MA 01605.

Protein kinases are largely responsible for carrying metabolic signals that promote the transcription of nuclear genes. We screened nuclear extracts of cultured cell lines for previously uninvestigated kinases that might phosphorylate transcription factors and regulate their activity. We identified a novel, regulated, soluble kinase in nuclear extracts from several different eukaryotic cell lines. The kinase migrated in sodium dodecyl sulfate-containing polyacrylamide gels as a multiplet with apparent molecular mass 95 kDa and was assayed by autophosphorylation of nitrocellulose-blotted, renatured enzyme. This assay selected for kinases which could renature and autophosphorylate after gel electrophoresis, which simplified the problem of too many activities in nuclear extracts. Serum or forskolin stimulation of serum-starved A431 cells increased the autophosphorylation of the 95 kDa multiplet, which was in turn correlated with an increase in substrate-directed phosphorylation. Stimulation was very rapid, taking place within 6 minutes. Of several protein substrates tested, only myelin basic protein was phosphorylated. Autophosphorylation was on serine and threonine residues, not on tyrosine, and substrate phosphorylation was only on serine. Autophosphorylation was completely blocked by treatment of serum-starved cells with transforming growth factor β-1 (TGFβ-1). The kinase was partially purified from HeLa nuclear extract; it was not significantly present in HeLa cytosolic extract and it is apparently not significantly related to RSK or MAP kinases. It is associated with a large protein complex (M_r > 1,500,000 by gel filtration) of unknown biological function. While the physiological substrates for this kinase are also unknown, the observation that its reversible phosphorylation is positively regulated by serum and forskolin, and negatively regulated by TGFβ-1, suggests that it may have a role in cell cycle control or in oncogenic transformation.

BZ 320 SIGNALLING IN A PLANT PATHOGENIC FUNGUS: ISOLATION OF A NOVEL, SPORE GERMINATION SPECIFIC PROTEIN KINASE GENE FROM COLLETOTRICHUM TRIFOLII, Dickman, Martin B., Department of Plant Pathology, University of Nebraska, Lincoln, NE 68583

Colletotrichum trifolii, a fungal phytopathogen, causes anthracnose disease of alfalfa. Extensive monoculture plantings of uniform genotypes of alfalfa has resulted in physiological specialization of this fungus. Thus, distinct races of the pathogen continually evolve which are pathogenic on previously resistant cultivars of alfalfa. The biochemical and molecular bases underlying race-cultivar, specificity are not understood. We are interested in how the fungus perceives and responds to plant cues during the early stages of pathogenesis. Specifically we are studying protein phosphorylation in the fungus. As the presence of protein kinases in phytopathogenic fungi has generally not been investigated, we have used standard gene cloning methodologies (e.g. dual oligonucleotide homology probing, PCR) to establish at the DNA level the presence of protein kinase-like genes in the fungus. Using an RNA-PCR method, we have shown that specific protein kinase-like transcripts are made during disease development. In vitro phosphorylation studies coupled with pharmacological reagents have suggested race specific and cultivar induced specific protein kinase activity, as well as an essential role for calmodulin during infection structure (appressorium) development. During the course of these studies, we have isolated a protein kinase-like gene with unusual structural features. While the catalytic domain possesses most of the conserved features of the protein kinase serine/threonine family, the regulatory region has none of the hallmark motifs commonly found in the 5' end. We found three elements of particular interest, a bZIP region, a proline rich area, and glutamine rich region. Moreover, primer extension analysis has indicated that this gene is primarily expressed during spore germination., an essential process in fungal development and pathogenesis. Characterization of this gene will be presented.

BZ 321 CLONING OF TWO PUTATIVE SERINE/THREONINE KINASES WITH HOMOLOGY TO S. cerevisiae CDC15, Jacqueline Doody, Liliana Attisano, Fernando Lopez-Casillas, Alejandro Zentella and Joan Massague, Department of Cell Biology and Genetics and Howard Hughes Medical Institute, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

We report the cloning of two cDNAs from Rat-1 cells encoding putative protein serine/threonine kinases. The open reading frames are approximately 1450bp in length which code for proteins of 58kD. These proteins are 96% identical within the kinase domain and 72% identical overall. A mouse homologue which has also been cloned in our lab is 98% identical to the kinase region of one of the rat clones. The kinase region shows consensus sequences characteristic of protein serine/threonine kinases.

Northern analysis of Rat-1 RNA revealed the presence of messages for both kinases at 3kb. These cDNAs also hybridized with mRNA species in mouse, mink and human cell lines of various tissue origins indicating a broad distribution among mammals. A survey of Genbank revealed that the S. cerevisiae CDC15 gene, required for completion of M phase in the cell division cycle, displayed the closest similarity to these two mammalian kinases (40% identity within the kinase region). The similarity of these cloned kinases to yeast CDC15, their conservation in several tissues of various mammalian species and the fact that two clearly related kinases exist in the same cells would indicate their importance in cellular processes, possibly cell division.

BZ 322 Posttranslational modification of α-PKC

I. Filipuzzi and R. Imber, Molekulare Tumorbiologie, Laboratorien Frauenspital, Schanzenstrasse 46, CH-4031 Basel, Switzerland

We have previously shown that the α-isoform of protein kinase C (α-PKC) is synthesized as a presumably inactive precursor which is then rapidly phosphorylated yielding the mature enzyme. The precursor has been postulated to represent an inactive molecule which is converted to the active form by phosphorylation. As the precursor does not undergo autophosphorylation, it is suggested that a trans-phosphorylation by an α-PKC-specific kinase is responsible for its phosphorylation and concomitant activation.

Here, we present a study where posttranslational phosphorylation of α-PKC was examined. α-PKC expression in E. coli has been found to generate the unphosphorylated precursor. The bacterially produced molecule was used as a substrate to identify protein kinases able to phosphorylate the α-PKC precursor. Furthermore, using deletion analysis and site-directed mutagenesis the phosphorylation site on the α-PKC molecule has been mapped. The impact of the transphosphorylation on α-PKC activity will be discussed.

BZ 323 p70^{s6k} SUBSTRATE RECOGNITION DETERMINANTS AND AUTOINHIBITORY DOMAIN: INHIBITORY PEPTIDES

Horst Flotow* & George Thomas. Friedrich Miescher Institut, P.O. Box 2543, 4002 Basel Switzerland.

Stimulation of quiescent cells in culture to proliferate leads to the coordinate activation of a number of biochemical processes culminating in DNA synthesis and cell division. One of the early obligatory steps in the mitogenic response is the two- to three-fold increase in the rate of protein synthesis, which is controlled at the level of initiation. This increase is accompanied by the phosphorylation of a number of specific proteins of the translational apparatus, one of which is the 40S ribosomal protein S6. Following mitogenic stimulation five moles of phosphate are incorporated into S6. The mitogen-activated p70^{s6k} responsible for carrying out this phosphorylation has been purified and cloned in our laboratory. It's optimal substrate recognition determinants have recently been determined to be (K/R)-R-X-R-X-X-S'-X (Flotow & Thomas, 1992). Using this information, synthetic peptides have been made which can act as competitive inhibitors of S6 phosphorylation to further investigate its role in the control of protein synthesis and cell proliferation. The results obtained suggest that some of the substrate peptides are relatively good inhibitors of S6 phosphorylation with K_i's approximately equal to their apparent K_m's. In addition to these substrates, peptides based on regions of p70^{s6k}, which appear to represent autoinhibitory domains have also been tested. Thus far, one region of the kinase with such properties has been localized. It contains the sites of phosphorylation required for activation of p70^{s6k} (Ferrari et al.,1992), and synthetic peptides containing this domain do not serve as a substrates for the enzyme but inhibit the kinase with a K_i of 30 μM or less.

References:

Flotow, H. & Thomas, G. (1992). J.Biol. Chem. 267: 3074-3078.
Ferrari, S. et al. (1992). PNAS 89: 7282-7286.

*Supported by a long term fellowship from the Human Frontiers in Science Program Organization

BZ 324 CHARACTERIZATION OF A CALMODULIN KINASE FROM EMBRYONIC AXES. Alicia Gamboa de Buen and Estela Sánchez de Jiménez. Departamento de Bioquímica, Facultad de Química, Universidad Nacional Autónoma de México, C.P. 04510, México, D.F.

Calmodulin (CaM) is a small protein that mediates many of the physiological effects of calcium. Phosphorylation of calmodulin is known to take place under physiological conditions both in serine and tyrosine residues. At present the functional meaning of these modifications is not fully understood. Some enzyme activities, however, are known to be modified by phosphorylation of CaM. The aim of this work was to isolate and characterize maize CaM-kinase in order to gain knowledge in the meaning of CaM phosphorylation as regulator of key metabolic pathways.

Postribosomal extracts of maize embryonic axes were used as the source of enzyme. The purification procedure employed sequential column chromatographies of DEAE-sephacell, phenyl-sepharose, CaM-agarose and TSKG 4000 SW. SDS-PAGE analysis of the purified enzyme showed two main peptides of 48 and 36 kDa. This enzyme phosphorylates calmodulin from different -- animal and plant sources. This reaction is inhibited by calcium and polylysine but not by polyamines. -- Further, the enzyme is able to autophosphorylate in a non-dependent Ca^{++} manner. Polylysine enhances -- this activity. Other ions and specific kinase inhibitors were also tested. A possible regulatory role of the enzyme on CaM function will be discussed.

This work was partially supported by CONACYT Grant No. 0666-N9108 and PADEP DFQ92242. AGB holds a fellowship from DGAPA, UNAM.

BZ 325 RECONSTITUTION OF A PHEROMONE INDUCED KINASE CASCADE IN S. CEREVISIAE: ACTIVATION OF THE FUS3 KINASE BY THE STE7 KINASE IN VITRO

Anton Gartner[1], Zhou-qing Zhou[2], Gustav Ammerer[1] and Beverly Errede[2]. [1]Institut fuer Allgemeine Biochemie, University of Vienna 1030 Vienna and [2]Department of Chemistry, University of North Carolina, Chapel Hill NC27599.

Pheromone stimulated haploid yeast cells undergo a differentiation process that allows them to conjugate. Transmission of the intracellular signal involves threonine and tyrosine (T180 and Y182) phosphorylation of the Fus3 and Kss1 kinases which are members of the MAP kinase family. FUS3/KSS1 phosphorylation, like all other responses to pheromone, depends on two additional kinases encoded by the *STE7* and *STE11* genes. A provisional order of the pathway is established in which STE11 acts before STE7 and FUS3. We further show that STE7 is a dual specificity kinase which modifies Fus3 in vitro at the appropriate sites thereby potentiating its activity. These data together with previously published genetic data, strongly suggest that STE7 is the physiological activator of FUS3. Recent indications that MAP kinase activators belong to the STE7 class of protein kinases suggest that signal transduction pathways in many if not all organisms use homologous kinase cascades.

BZ 326 CELLULAR STUDIES ON THE REGULATION OF HMG-COA REDUCTASE BY THE AMP-ACTIVATED PROTEIN KINASE

John G.Gillespie, Susan Dale and D.Graham Hardie, Department of Biochemistry, The University, Dundee, DD1 4HN.

HMG-CoA reductase catalyses the principal regulatory step in the biosynthesis of cholesterol and other isoprenoid compounds, namely the conversion of HMG-CoA to mevalonate. This enzyme is subject to multivalent control mechanisms including phosphorylation. Studies in this laboratory have shown that an AMP-activated protein kinase phosphorylates and inactivates HMG-CoA reductase. In addition this kinase phosphorylates and inactivates acetyl-CoA carboxylase which catalyses the principal regulatory step of fatty acid biosynthesis, thereby providing a mechanism to co-ordinately control fatty acid and cholesterol biosynthesis when their common precursor, acetate, is limiting. It has been shown previously that incubation of isolated hepatocytes with fructose leads to elevation of AMP and activation of the AMP-activated protein kinase. This treatment causes marked inactivation of HMG-CoA reductase. Immunoprecipitation from the microsomal fraction of ^{32}P-labelled hepatocytes leads to 2.6 fold increase in the phosphorylation of the 100kDa subunit of HMG-CoA reductase. Successive digestion of this ^{32}P-labelled subunit with cyanogen bromide and endoproteinase Lys-C confirms that ser-871, the site phosphorylated in cell free assays by the AMP-activated protein kinase, was the only site phosphorylated under these conditions. Our long term aim is to determine the role of this phosphorylation site using site-directed mutagenesis. Studies to determine if HMG-CoA reductase and the AMP-activated protein kinase are co-ordinately expressed are currently underway at the moment. We are examining this in three cell lines which express different levels of HMG-CoA reductase - CHO-K1, UT-2, and UT-1 - the latter two cell lines are mutants derived from CHO-K1 cells. CHO-K1 cells express normal levels of HMG-CoA reductase, UT-2 cells express very low levels, and UT-1 cells, when grown on lipoprotein deficient serum and compactin , a competitive inhibitor of HMG-CoA reductase, express up to 500 fold more HMG-CoA reductase than CHO-K1 cells. Using these cell lines we shall establish whether the level of AMP-activated protein kinase expression changes with that of HMG-CoA reductase.

BZ 327 A *raf* HOMOLOG INVOLVED IN A SIGNAL TRANSDUCTION PATHWAY THAT REGULATES *C. ELEGANS* VULVAL DEVELOPMENT,

Andy Golden*, Min Han#, and Paul W. Sternberg* ,*Howard Hughes Medical Institute and Division of Biology, 156-29, California Institute of Technology, Pasadena, CA 91125, #Dept. MCD Biology, University of Colorado at Boulder, Boulder, CO 80309

Vulval induction in the nematode *C. elegans* involves an inductive signal originating from the anchor cell in the somatic gonad. Three of six vulval precursor cells within the ventral hypodermis respond to this inductive signal to generate the cells that form the vulva. Mutations in a number of genes disrupt this inductive process such that vulvaless (Vul) or multivulval (Muv) animals develop. Molecular cloning has revealed that some of these nematode genes encode homologs of mammalian signal transduction molecules. The *lin-3* gene is predicted to encode a transmembrane protein with a single EGF repeat and is expressed in the anchor cell. The *let-23* gene encodes a receptor tyrosine kinase of the EGF receptor family and the *let-60* gene encodes a *ras* homolog. Mutations in these genes result in Vul animals. Dominant, gain-of-function *let-60* alleles result in Muv animals. By epistasis experiments, these genes function in a linear pathway to induce vulval differentiation.

We molecularly cloned the *C. elegans raf-1* homolog to examine whether it functions in vulval induction and other aspects of nematode development. The predicted amino acid sequence of the cDNA and genomic sequences revealed 80% sequence similarity with the kinase domain of the human *c-raf-1* gene product. Conserved regions 1 (CR1) and 2 (CR2) were also evident in the amino-terminal regulatory domain. The genomic map position of the nematode *raf-1* homolog corresponded to the map position of another Vul gene, *lin-45*. Injection of the genomic *raf-1* sequences rescued the Vul phenotype of *lin-45* mutant animals. In addition, the molecular defect in the *lin-45* allele, *sy96*, resides within the *raf-1* gene. These data suggest that the *lin-45* gene encodes the *C. elegans raf-1* homolog and that mutations in this gene disrupt *C. elegans* vulval development. Epistasis experiments with dominant *let-60* alleles suggest that *lin-45/raf-1* acts downstream of the *let-60* gene.

To further characterize the effects of *lin-45/raf-1* mutations in development, we are generating transgenic animals expressing mutant variants of this gene. We intend to characterize the phenotypic consequences in such animals as well as use these animals for extragenic suppression screens to identify other gene products that act in this signalling pathway during nematode development.

BZ 328 THE STIMULATION OF KINASES WHICH ARE DISTINCT FROM KNOWN ISOTYPES OF PROTEIN KINASE C BY PHORBOL ESTERS WITH DIFFERING BIOLOGICAL ACTIVITIES. Phil C Gordge, W Jonathan Ryves, Pawan Sharma, A Tudor Evans and Fred J Evans. The Department of Pharmacognosy, The School Of Pharmacy, University of London, 29-39 Brunswick Square, London, WC1N 1AX, U.K.

Diterpene ester toxins of the tigliane, ingenane and daphnane classes possess potent biological activities, the most notable of which are their acute pro-inflammatory and their tumour promoting properties when applied chronically. Recent analysis of the pro-inflammatory response induced by the daphnane orthoester, Resiniferatoxin (Rx), indicates that *this irritancy* exhibits features of a mixed aetiology, which is comprised of both neurogenic and phorbol ester components [1]. Furthermore, we have isolated a novel Ca^{2+}-inhibited kinase activity from human mononuclear cells [2] and mouse peritoneal macrophages [3], which is stimulated by Rx to a greater extent than 12-O-tetradecanoylphorbol-13-acetate (TPA) and which is distinct from known isotypes of protein kinase C (PKC), which has been defined as the tumour promoting phorbol ester receptor [4].

Other *in vitro* phosphorylation studies have indicated that phorbol esters with different biological activities are capable of selective activation/non-activation of purified PKC isotypes [5]. We have therefore studied the hyperplasiogenic and pro-inflammatory responses induced by a series of phorbol esters and the *in vitro* kinase stimulation profile of these compounds. This suggests that irritancy may be a better correlation for kinase activation properties than tumour promotion, and that the biochemical mechanisms of action of the phorbol esters may partly involve the activation of kinases which are distinct from known isotypes of PKC.

References: [1] Evans,A.T., *et al*, (1992) *J.Pharm.Pharmacol.* **44** 361.
[2] Ryves,W.J., *et al*, (1989) *FEBS.Letts.* **245** 159.
[3] Evans,A.T., *et al*, (1990) *FEBS.Letts.* **267** 253.
[4] Castagna,M., *et al*, (1982) *J.Biol.Chem.* **257** 7847.
[5] Evans,F.J., *et al*, (1991) *Biochem.Soc.Trans.* **19** 397.

BZ 329 PURIFICATION AND CLONING OF A MAP KINASE KINASE FROM RABBIT SKELETAL MUSCLE. Lee M. Graves, Brian Potts. Barbara Glidden, Rony Seger, James E. Weiel, Santosh Kumari and Edwin G. Krebs, Department of Pharmacology, University of Washington, Seattle,WA 98195.

A 44 kDa protein kinase capable of phosphorylating and activating recombinant Erk-2 (MAP kinase) was purified several thousand fold from rabbit skeletal muscle. Two forms of the Map kinase kinase (MAPKK) were observed during ion exchange chromatography, possibly due to different phosphorylation states or isoforms of the enzyme. MAPKK was found to autophosphorylate on serine,threonine and tyrosine demonstrating that this enzyme belongs to the class of dual specificity kinases. Peptide sequence obtained from trypsin digestion of the enzyme indicated that this kinase was similar to the yeast signalling kinases Ste-7 and Byr-1. Polymerase chain amplification was used to facilitate the isolation of the full length clone of the skeletal muscle kinase. Primary sequence comparisons showed close homology to the Map kinase kinase cloned from a human T-cell library. In addition, sequence analysis demonstrated regions of similarity to other protein kinases, in particular the yeast kinases Ste-7 and Byr -1. These results suggest a high degree of conservation of signal transduction pathways from yeast to mammalian cells.

BZ 330 PROTEIN KINASE C ISOENZYMES IN MOUSE EPIDERMIS. ACTIVATION, INHIBITION, DOWN-REGULATION AND SUBSTRATES, Michael Gschwendt, Hanno Leibersperger, Walter Kittstein and Friedrich Marks, German Cancer Research Center, Im Neuenheimer Feld 280, W-6900 Heidelberg, Germany

The classical system for the investigation of tumor promotion is mouse skin. Tumor promoting phorbol esters, such as TPA, are known to activate protein kinase C (PKC), a family of isoenzymes that plays a key role in intracellular signal transduction. Therefore, it is thought that the effect of TPA on many cellular processes, including tumor promotion, is mediated by activation of PKC.

Murine epidermis contains the Ca^{2+}-responsive PKC isoenzymes α and β as well as the Ca^{2+}-unresponsive isoenzymes δ, η and ζ. Just traces of PKCϵ are found. This is demonstrated by the use of specific antibodies. PKCζ predominates in the cytosol and PKCδ and η are found mainly in the particulate fraction. Whereas epidermal PKCα,β,δ and η are down-regulated by treatment of mouse skin with TPA or bryostatin 1 for 18 h, PKCζ is not affected by this treatment. Furthermore, contrary to other PKC isoenzymes, cytosolic PKCζ is not translocated to the particulate fraction upon treatment of the animals with TPA. In this context it might be of interest that PKCζ not only lacks the C-2 region, like the other Ca^{2+}-unresponsive isoenzymes, but also the second cysteine-rich region, which we consider to be important for the high affinity binding of TPA. Various PKC isoenzymes differ considerably in their sensitivity towards the staurosporine derivative K252a, whereas staurosporine exhibits a similar inhibitory capacity for all isoenzymes. Two epidermal proteins (50 kDa and 37 kDa) are phosphorylated by Ca^{2+}-responsive as well as Ca^{2+}-unresponsive isoenzymes.

BZ 331 PARTIAL PURIFICATION AND CHARACTERISATION OF THE INSULIN-STIMULATED ACETYL-CoA CARBOXYLASE KINASE FROM RAT EPIDIYMAL FAT PADS. K. Heesom, K. Moule & R.M. Denton.
Dept. Biochemistry, School of Medical Sciences, University Walk, Bristol, BS8 1TD, U.K.

Activation of acetyl-CoA carboxylase in adipose tissue by insulin is associated with an increase in the phosphorylation of the enzyme on at least two separate serine residues. One of these serine residues (Ser-29) is a substrate for casein kinase-II. The other phosphoserine remains to be identified, but is known to occur in a tryptic peptide termed the I-site. As phosphorylation of Ser-29 has no apparent effect on the activity of acetyl-CoA carboxylase, it is likely that phosphorylation of the I-site is responsible for the increase in the activity of the enzyme in response to insulin. The insulin-stimulated kinase responsible for phosphorylation of the I-site copurifies on Mono Q anion exchange chromatography with CoA, a potent inhibitor of acetyl-CoA carboxylase [1,2]. Phosphorylation of acetyl-CoA carboxylase by the kinase converts the carboxylase to an active, CoA insensitive form.

Further purification of the kinase has been achieved using hydroxyapatite and Superose 12 chromatography. This kinase preparation is unreactive towards a number of substrates, including myelin basic protein, but does phosphorylate the 32 and 8 residue peptides derived from ribosomal S6. The substrate specificity of the kinase differs from those of $p90^{rsk}$ and $p70^{S6k}$ and appears to be immunologically indistinct from these well characterised S6 kinases. The activity of the kinase is unaffected by incubation with protein phosphatase 2A. The acetyl-CoA carboxylase kinase and S6 peptide kinase activities also copurify on phenyl-superose and Mono S chromatography and may therefore represent a single novel insulin-stimulated protein kinase.

[1] Borthwick, A.B., Edgell, N.E. & Denton, R.M. (1990) Biochem. J. **270**, 795-801.
[2] Moule, S.K., Edgell, N.E. & Denton, R.M. (1992) Biochem. J. **283**, 35-38.

BZ 332 Autophosphorylation sites on the Catalytic Subunit of cAMP-dependent Protein Kinase.

Friedrich W. Herberg, Wes Yonemoto and Susan S. Taylor
Department of Chemistry, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0654, USA

cAMP-dependent protein kinase is one of best characterized members in the diverse family of protein kinases. The inactive tetrameric holo enzyme contains two regulatory (R) and two catalytic (C) subunits. The complex is dissociated in response to nanomolar molar concentrations of the second messenger cAMP, yielding an R_2cAMP_4 dimer and two active monomeric C-subunits.

The C-subunit purified from skeletal muscle (type I) and heart (type II) and the recombinant protein, overexpressed in E. coli have similar enzymatic and biochemical properties. Each could be separated into two or three different isoelectric forms using Mono S ion exchange chromatography; however the physical basis for these differences in the catalytic subunit isozymes was never determined. In contrast to the mammalian C-subunit, the recombinant protein is not myristoylated at the N-terminus. Electrospray mass spectrometry established that the three isozymes separated from the recombinant C-subunit are due to differences in autophosphorylation. The mammalian enzyme can be separated into two isozymes that show identical mass corresponding to two phosphates and one myristoyl group.

The autophosphorylation sites within the recombinant C-subunit are Ser 10, Ser 139, Thr 197 and Ser 139, while only Thr 197 and Ser 338 are thought to be phosphorylated in the mammalian enzyme. Thr 197 is extremely resistant to removal by phosphatases. Its interaction with His 87, Arg 165 and Lys 189 in the crystal structure explains this resistance and suggests that the phosphorylation of Thr 197 is essential for the active conformation of the enzyme. Myristoylation at the amino-terminus contributes structural stability to the enzyme and does not convey membrane-association properties. The recently solved crystal structures of the C-subunit in complex with an inhibitory peptide (PKI_{5-24}) in the presence or absence of MgATP have been used to elucidate the problems discussed above. These results have been extended to other members of protein kinase family.

BZ 333 MULTIPLE LEVELS OF PIM-1 KINASE REGULATION IN HEMATOPOIETIC CELLS, Debra S. Hoover, Denise Wingett, Raymond Reeves and Nancy S. Magnuson, Departments of Genetics and Cell Biology, Biochemistry and Microbiology, Washington State University, Pullman, Wa. 99164.

The Pim-1 protein is a serine-threonine kinase expressed predominantly in hematopoietic tissue. A role for Pim-1 in signal transduction has been implicated. Increases in *pim-1* mRNA levels are observed upon induction with cytokines and phorbol esters. Using antibodies generated to the recombinant human Pim-1 we have studied the pattern of Pim-1 expression in both primary and cultured cell lines. We find the protein levels do not always parallel the kinetics of mRNA induction. These differences in expression patterns suggest post-transcriptional regulatory mechanisms may be operating under our induction conditions. We will describe and discuss the mechanisms thought to be involved in Pim-1 regulation. This work was funded in part by NIH grant AI-26356 and USDA grant 91-37206-6867.

BZ 334 MOLECULAR CLONING OF A MAMMALIAN MEMBER OF THE MAP KINASE ACTIVATOR FAMILY. Rowland T Hughes[1], Beverly Yashar[2], Karen Yee[1], Gail Bruns[1], Beverly Errede[2], Leonard I Zon[1]. [1]Department of Hematology/Oncology, Children's Hospital, Boston, MA, 02115. [2]Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina, 27599.

Phosphorylation and activation of mitogen activated protein (MAP) kinases are intermediary events in cascades of protein phosphorylation initiated by a variety of extracellular stimuli. Genetic studies of the protein kinases activated in the yeast mating signalling pathway indicate that the protein kinase STE7 activates a MAP kinase homolog, FUS3. Recently, a MAP kinase activator has been purified biochemically from Xenopus oocytes and shown to have significant amino acid similarity to STE7. We have isolated Xenopus cDNA clones with predicted amino acid sequence that are homologous to STE7, but distinct from the activator previously described. Using the Xenopus cDNA as a probe, we screened a murine 6.5 day embryo library and a murine erythroleukemia library, and obtained cDNAs which are highly homologous to the Xenopus cDNA. A homologous human cDNA has also been isolated from an umbilical vein endothelial cell library. The predicted amino acid sequences of the murine and Xenopus clones are 93% identical. Optimal alignment was obtained by introducing a 105 nucleotide gap in the murine cDNA sequence, indicating possible alternative processing of the gene, or evolutionary divergence. The predicted molecular weight of the murine product is 46kD. Northern blot analysis of multiple murine tissues, including hematopoietic cell lines of all lineages, indicates that this gene is ubiquitously expressed, with the highest levels found in brain and skeletal muscle. PCR analysis of DNAs from mouse/human or hamster/human cell hybrids using oligonucleotides unique to the 3' untranslated region of the human cDNA indicates that the human gene is localized to chromosome 17. Functional studies of this novel protein kinase await the production of specific antisera and synthesis of recombinant protein. The study of the family of MAP kinase activators will undoubtedly be relevant to cell signalling in response to extracellular signals.

BZ 335 PURIFICATION OF A PROTEIN KINASE/LIPID KINASE COMPLEX ESSENTIAL FOR VACUOLAR PROTEIN SORTING IN YEAST. Greg Huyer, Jeffrey H. Stack, Kaoru Takegawa, Peter V. Schu, and Scott D. Emr, Division of Cellular and Molecular Medicine, Howard Hughes Medical Institute, University of California, San Diego, School of Medicine, La Jolla, CA 92093-0668

Protein sorting to the yeast lysosome-like vacuole is a complex process involving greater than 40 *vps* (vacuolar protein sorting) genes in *S. cerevisiae*. The *VPS15* gene encodes a 1455 amino acid myristoylated phosphoprotein that has significant homology to the serine/threonine family of protein kinases. The *VPS34* gene encodes an 875 amino acid protein that is the yeast homologue of the catalytic subunit of the mammalian phosphatidyl-inositol 3-kinase (PI3-kinase). Mutations in conserved protein kinase residues in Vps15p lead to complete missorting of a soluble vacuolar hydrolase, carboxypeptidase Y (CPY). Similarly, mutations in the *VPS34* gene that inactivate PI3-kinase activity also cause complete missorting of CPY. Genetic evidence suggested that Vps15p and Vps34p interact: Overproduction of Vps34p in *vps*15 kinase domain point mutants suppresses the *ts* growth and vacuolar protein sorting defects associated with the *vps*15 mutant strains. *vps*15 null mutants cannot be suppressed by the overproduction of Vps34p; thus, Vps34p cannot bypass the cells' requirement for Vps15p. A physical association between Vps15p and Vps34p was directly demonstrated by immunoprecipitation experiments: In both native immunoprecipitates and in chemically cross-linked extracts, Vps15p and Vps34p are co-immunoprecipitated with antisera specific for either protein. Fractionation studies showed that this complex is peripherally associated with the cytosolic face of a membrane compartment. To gain some insight into the molecular mechanisms by which this protein complex regulates vacuolar protein sorting, efforts to purify the complex were undertaken. With purified complex, we hope to better define the biochemical properties of these enzymes. Purification can also facilitate the identification of the biological regulators and substrates of these enzymes. Specifically, the putative protein kinase activity of Vps15p may regulate the PI3-kinase activity of Vps34p; in turn, a phosphatase may be involved to complete the regulatory loop. Vps15p itself may also be regulated by another factor that could be identified through these studies. This enzyme complex may be involved in a PI signaling cascade that triggers downstream events in vacuolar protein trafficking; by studying the molecules involved in generating the signaling event we may learn how this signal is transduced to cause, for example, vesicle formation or fusion.

BZ 336INTERACTION CLONING OF PROTEIN KINASE C SUBSTRATES, Susan Jaken, Christine Chapline, Katrina Ramsay, and Theresa Klauck, The W. Alton Jones Cell Science Center, Lake Placid, NY 12946

Immunocytofluorescence studies have demonstrated that α-protein kinase C (PKC) is concentrated in cell-cell and cell-substratum adhesion sites in renal proximal tubule epithelial cells and REF52 fibroblasts, respectively. Possibly, α-PKC is targeted to these specific areas via interactions with resident proteins. An overlay assay approach identified 2 major PKC binding proteins in REF52 cell extracts. The potential significance of these proteins in targeting PKC to focal contacts was suggested because these binding proteins were not detected in extracts of SV40-REF52 cells in which α-PKC is not localized to focal contacts. Comparison of the properties of REF52 cell PKC binding proteins and PKC substrates indicated that the major binding proteins were also substrates. We therefore used the overlay assay to screen expression libraries in order to identify PKC binding proteins and substrates. Two clones isolated from a rat kidney λgt11 library have now been characterized. Clone 35A is the rat homologue of mouse F52 protein. F52 contains the phosphorylation sequence found in the major PKC substrate, MARCKS. Clone 35H has significant homology to regions of β-adducin, including a putative PKC phosphorylation site. Phosphorylation of the proteins expressed by clones 35A and 35H attenuates PKC binding, indicating that PKC interactions with these proteins are regulated by phosphorylation. The overlay assay screening approach appears to be a useful method for cloning and identifying novel PKC substrates.

BZ 337 MICROINJECTED ANTIBODIES TO PHOSPHO-TYROSINE INHIBIT INSULIN-STIMULATED SERINE PHOSPHORYLATION OF RIBOSOMAL PROTEIN S6, In-Soon Kim and A. Raymond Frackelton, Jr., Department of Medicine, Brown University and Roger Williams Medical Center, Providence, RI 02908

We attempted to gain direct evidence in intact cells concerning the role of tyrosine phosphorylation in the cascade of biochemical events whereby numerous growth factors and cellular activators stimulate serine phosphorylation of ribosomal protein S6. As an approach to this problem, antibody to phosphotyrosine and, as a positive control, neutralizing antibody to insulin receptor tyrosine kinase were each microinjected into BALB/C 3T3 and CHO cells. Each antibody impaired the ability of insulin to cause serine phosphorylation of ribosomal protein S6, but only the phosphotyrosine antibody reduced basal S6 phosphorylation. In contrast, antibody to phosphotyrosine appeared to increase insulin-dependent tyrosine phosphorylation of a 180-kDa protein, the major cellular substrate of the insulin receptor kinase, suggesting that the antibody did not impair the receptor's kinase activity. These results with intact cells support numerous in vitro studies implicating non-receptor tyrosine-phosphorylated proteins in the biochemical pathway(s) leading to S6 phosphorylation.

BZ 338$cPLA_2$ IS PHOSPHORYLATED AND ACTIVATED BY MAP KINASE. JOHN L. KNOPF‡, LIH-LING LIN‡*, ALICE Y. LIN‡, MARKUS WARTMANN¶, ALPNA SETH¶, AND ROGER J. DAVIS¶# . ‡GENETICS INSTITUTE, INC. CAMBRIDGE, MASSACHUSETTS 02140. #HOWARD HUGHES MEDICAL INSTITUE¶ PROGRAM IN MOLECULAR MEDICINE DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY UNIVERSITY OF MASSACHUSETTS MEDICAL SCHOOL WORCESTER, MA 01605.

Upon treatment of cells with agents that stimulate the release of arachidonic acid, the phosphorylation of $cPLA_2$ is increased suggesting that $cPLA_2$ phosphorylation is causally associated with its activation. Here we report that purifed $cPLA_2$ is a substrate for the mitogen activated protein kinase, MAP kinase. Phosphorylation of $cPLA_2$ by MAP kinase increased the enzymatic activity of $cPLA_2$ and resulted a decreased mobility on SDS-PAGE. Phosphopeptide mapping of MAP kinase-phosphorylated $cPLA_2$ revealed three major phosphopeptides which co-migrated with ^{32}P labeled $cPLA_2$ phosphopeptides derived from TPA treated CHO cells. A consensus phosphorylation site (Pro-Leu-Ser-Pro) for MAP kinase was found at amino acid position 505. Mutation of the serine residue to an alanine resulted in a mutant protein which was no longer a substrate for MAP kinase *in vitro*. Unlike CHO cells overexpressing wild-type $cPLA_2$, the SA505-$cPLA_2$ showed little or no increase in agonist induced arachidonic acid release as compared with parental CHO cells.

BZ 339INHIBITION OF PK-C LEADS TO A POTENT PRIMING SIGNAL FOR THE RESPIRATORY BURST IN HUMAN EOSINOPHILS. Leo Koenderman, Tjomme van de Bruggen, Jan A.M. Raaijmakers, Jan-Willem J. Lammers, Anton TJ Tool and Arthur J. Verhoeven. Depts. Pulmonary Diseases, University Hospital Utrecht, PO Box 85500, 3508 GA Utrecht, and Bloodcell Chemistry, CLB, Amsterdam, The Netherlands.

Opsonized particles, such as serum-treated zymosan (STZ), are poor activators for eosinophils isolated from the blood of normal individuals. However, after priming with cytokines such as IL-3, IL-5, and GM-CSF, which by itself do not activate effector mechanisms in eosinophils, addition of opsonized particles to these cells leads to a very potent activation signal. Investigating the possible mechanism of this priming reaction, we studied the role of changes in $[Ca^{2+}]_i$ and the role of PK-C in this priming reaction. Changes in $[Ca^{2+}]_i$ are not involved because the priming reaction is not influenced by depletion of the cells from Ca^{2+} (resting $[Ca^{2+}]_i$< 10nM). When the effect of the PK-C inhibitor staurosporine was tested, it was found that addition and removal of staurosporine already leads to a potent priming signal. The dose response curve of the priming reaction by staurosporine superimposes the inhibition of the PMA-induced respiratory burst. Because staurosporine is a potent but not very specific inhibitor of PK-C, we applied the newly developed staurosporine analogues CGP 41251 and CGP 44800, that are specific inhibitors of PK-C. Again, addition and removal of these analogues resulted in dose dependent priming of the respiratory burst, which superimposed the inhibition of the respiratory burst induced by PMA. This study demonstrates that a short-term inhibition of PK-C leads to priming of the respiratory burst in human eosinophils. Therefore, modulation of the protein kinase/phosphatase balance might be a underlying mechanism in priming reactions in human granulocytes.

BZ 340 NOVEL PROTEIN KINASE ACTIVITIES IN CELLS INFECTED WITH VACCINIA VIRUS, David P. Leader* and Georges Beaud†, *Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, U.K., and †Institut Jacques Monod, Paris , France.

In cells infected with vaccinia virus there occurs phosphorylation of ribosomal proteins S2 and S13, proteins that have are not known to be phosphorylated in uninfected cells. Ribosomal protein S2 has four phosphorylation sites (three seryl and and one threonyl), whereas ribosomal protein S13 has a single phosphoserine. Protein kinase activities were detected in infected cells and these were capable of similar multiple phosphorylation of ribosomal protein S2 and monophosphorylation of S13 *in vitro*.

We have now performed further purification of the ribosomal kinase activities that indicates distinct species are present. The post-ribosomal supernatant of HeLa cells was subjected to successive chromatography on columns of DEAE-cellulose, phosphocellulose, DNA-cellulose and α–casein-agarose. The S2- and S13 kinase activities comigrated on phosphocellulose and DNA-cellulose, and were associated with an α–casein kinase activity and a 34 kDa protein that was strongly phosphorylated *in vitro*. On α-casein-agarose chromatography the S13 kinase activity was eluted early as a homogeneous peak together with the 34 kDa phosphoprotein and before the α-casein kinase activity. On a glycerol density gradient it sedimented as a single peak at about 4.9S. In contrast, S2 kinase activity exhibited heterogeneity, part eluting in the region of the S13 kinase, and part at higher ionic strength. Glycerol density gradient analysis of the S2 kinase revealed forms sedimenting at 2.4S and 5.5S. ATP-agarose chromatography of the S2 kinase after the casein-agarose step resulted in an apparently homogeneous protein of 36 kDa.

Recent evidence suggests that the S2 kinase activity (at least) may derive from a viral protein kinase, the product of gene B1R, as the recombinant B1R protein kinase phosphorylates ribosomal protein S2 *in vitro*.

BZ 341 INHIBITION OF NEUTROPHIL NADPH OXIDASE ASSEMBLY BY MYRISTOYLATED INHIBITOR PEPTIDES OF PROTEIN KINASE C AND PROTEIN KINASE A, J.H.W. Leusen[1], D. de Bont[2], G.C.R. Kessels[1], M. Blom[1], R.M. Liskamp[2] and A.J. Verhoeven[1], [1]Centr. Lab. of the Neth. Red Cross Blood Transf. Service and Lab. of Exp. and Clin. Immunol, Univ. of Amsterdam, Amsterdam, the Netherlands; [2]Dept. of Organic Chem., Univ. of Leiden, Leiden, The Netherlands.

The superoxide-forming NADPH oxidase of human neutrophils is composed of membrane-bound and cytosolic proteins which, upon cell activation, assemble on the cell membrane to form the active enzyme. The cytosolic components include a 67-kDa protein (p67-*phox*) and a 47-kDa protein (p47-*phox*), which is a substrate for both PKC and PKA-mediated protein phosphorylation. Assembly of the oxidase in intact neutrophils can be induced by PMA, but not by dBcAMP. The importance of PKC activation after receptor-mediated cell activation is still controversial, mainly due to the lack of specific PKC inhibitors. We have tested a novel (nona)peptide inhibitor of PKC (resembling the autophosphorylation site of PKC), which is myristoylated at the N-terminus (Myr-NP). Myr-NP inhibited NADPH oxidase activation in electropermeabilized cells, both with PMA and the receptor agonist fMLP. However, a myristoylated peptide, designed to inhibit PKA-mediated phosphorylations (Myr-AP), had a similar inhibitory effect, although the PMA-induced protein phosphorylations were not affected. To further analyze the mechanism of inhibition, the effect of these peptides was also studied in a cell-free activation system containing neutrophil membranes, cytosol, GTPγS and low concentrations of SDS (100 μM). In this system, oxidase activation does *not* require protein phosphorylation. Both Myr-NP and Myr-AP inhibited NADPH oxidase activity in this assay completely (K_i's < 1 μM). The nonmyristoylated peptides were 10 times less effective. Preincubation of membranes with Myr-NP or Myr-AP and reisolation of the membranes resulted in a strongly reduced activity in the cell-free assay. Western blot analysis showed a diminished translocation to the membrane fraction of p47-*phox* and p67-*phox* in the presence of both peptides. We conclude that both Myr-NP and Myr-AP associate with neutrophil membranes and efficiently inhibit the assembly of the NADPH oxidase. Both peptides contain several positively charged amino acids. Indeed, a myristoylated peptide having several negatively charged residues was without effect on NADPH oxidase assembly, suggesting that the membrane surface charge is critical for the interaction between the membrane-bound and cytosolic components of the NADPH oxidase.

BZ 342 TENSIN, AN ACTIN-BINDING AND SH2-CONTAINING PROTEIN, IS ASSOCIATED WITH A SER/THR KINASE ACTIVITY. Su Hao Lo and Lan Bo Chen. Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA02115.

Tensin is a novel actin-binding 200 kD component of focal contacts. The cDNA deduced amino acid sequence contains LE(D)VL sequences known to be essential for IL-2/EPO signal transduction. It also reveals various domains sharing significant homology with cytoskeletal proteins (actin, catenin, actin-binding proteins) and proteins involved in signal transduction (somatolactin, BCR, IL-2/EPO receptor, IL-3 receptor, synapsin Ia, src homology 2 domain). It presents 10 consensus phosphorylation sites for tyrosine kinases and 50 potential phosphorylation sites for ser/thr kinases. Indeed, tensin is predominantly phosphoryated on serine and threonine residues in normal cells and becomes tyrosine-phosphorylated in RSV-transformed cells. In vitro assay shows tensin is phosphorylated by protein kinase C and cdc2 kinase. Most intriguingly, the recombinant tensin purified from the Baculovirus expression system remains associated with a kinase activity which phosphorylates tensin. Phosphoamino acid analysis reveals that serine and threonine residues are phosphorylated. This kinase activity remains associated with tensin under high salt (2M NaCl) or low pH (2.5) conditions during purification. When the epitope-tagged tensin is specifically eluted from an affinity column by synthetic peptides (9 residues), tensin retains the kinase activity. This phosphorylation activity is inhibited by kinase inhibitors such as lavendustin A, tyrphostin, Methyl 2.5-Dihydroxycinnamate, 2-hydroxy-5-(2,5-dihydroxybenzyle) aminobenzoic acid, but not by genistein. Our other studies have localized two actin-binding domains at the N-terminal region and demonstrated the functional SH2 domain at the C-terminal region. Taken togather, it suggests that tensin might be an integrator for signal transduction and the cytoskeleton via novel phosphorylation cascades.

BZ 343 THE RAF KINASE IS ASSOCIATED WITH THE T CELL RECEPTOR/CD3 COMPLEX, Christine Loh and Anjana Rao, Division of Tumor Virology, Dana Farber Cancer Institute and Department of Pathology, Harvard Medical School, Boston, MA. 02115

Protein kinases and phosphatases have been shown to play an important role in signal transduction. In the T cell, activation via the T cell receptor/CD3 complex results in rapid tyrosine phosphorylation of proteins, as well as subsequent increases in serine/threonine phosphorylation. It has been previously shown that the p59Fyn tyrosine kinase is associated with the T cell receptor complex. We confirm those earlier observations and further demonstrate that Raf, a serine/threonine kinase, is associated with the T cell receptor/CD3 complex in a resting T cell clone, Ar-5.

BZ 344 CHARACTERIZATION AND *IN VITRO* ASSAY OF RECOMBINANT *raf*-1, Susan G. Macdonald, Leah Conroy, Jonathan Driller, Robin Clark and Frank McCormick, Onyx Pharmaceuticals, Richmond, CA 94806.

Raf-1 is a ubiquitous cytoplasmic serine/threonine kinase which is activated by a variety of growth factors and appears to play a fundamental role in cell growth and differentiation. It is believed to be necessary for the function of *ras* and has recently been proposed to act upstream of MAP kinase kinase. The events directly leading to the activation of *raf*-1, however, are poorly understood. *Raf*-1 consists of an amino terminal region which contains a zinc finger domain that is homologous to those found in protein kinase C and N-chimaerin. The amino terminal region also contains a serine/threonine rich domain which may contain regulatory phosphorylation sites. The carboxy terminal region of *raf*-1 consists only of the kinase domain. Removal of the amino terminal region of *raf*-1 from its kinase domain results in constitutive activation of *raf*-1, thus identifying the amino terminal of *raf*-1 as a regulatory domain. In order to develop an *in vitro* system with which to identify potential activator(s) of the *raf*-1 kinase, we cloned epitope tagged versions of human *raf*-1 into baculovirus vectors and expressed the proteins in insect cells. The clones encoded full length and amino terminally truncated forms of *raf*-1 and versions of these proteins in which the lysine residue in the ATP binding site had been mutated. The epitope tags enabled us to easily purify large quantities of *raf*-1 from the cell lysates by immunoaffinity chromatography. We have assessed the catalytic activities of these proteins *in vitro* and are currently using the proteins to identify potential *raf*-1 activating factors. Results of these studies will be discussed.

BZ 345 CHARACTERIZATION OF THE PROTO-ONCOGENE *PIM-1*: KINASE ACTIVITY AND SUBSTRATE RECOGNITION SEQUENCE. Nancy S. Magnuson, Michael Friedmann, Mark S. Nissen, Debra S. Hoover and R. Reeves, Departments of Microbiology and Biochemistry, Washington State University, Pullman, WA 99164-4233.

The human *pim-1* proto-oncogene was expressed in *E. coli* as a GST-fusion protein and the enzymatic properties of its kinase activity were characterized. Likewise, a Pim-1 mutant lacking intrinsic kinase activity was constructed by site-directed mutagenesis (Lys ^{67}to Met) and expressed in *E. coli*. No kinase activity could be detected in *in vitro* assays with the mutant Pim-1 kinase. The wild-type Pim-1 kinase-GST fusion protein showed a pH optima of 7 to 7.5 and optimal activity was observed at either 10 mM $MgCl_2$ or 5 mM $MnCl_2$. Higher cation concentrations were inhibitory, as was the addition of NaCl to the assays. Previous work by this laboratory assaying several proteins and peptides showed histone H1 and the peptide Kemptide to be efficiently phosphorylated by recombinant Pim-1 kinase. In this study we examined the substrate sequence specificity of Pim-1 kinase in detail. Comparison of different synthetic peptide substrates showed Pim-1 to have a strong substrate preference for the peptide Lys-Arg-Arg-Ala-Ser*-Leu-Gly-Pro with an almost 6-fold higher specificity constant k_{cat}/K_m over the substrate Kemptide (Leu-Arg-Arg-Ala-Ser*-Leu-Gly). The presence of basic amino acid residues on the amino terminal side of the target Ser/Thr was shown to be essential for substrate recognition. Furthermore, phosphopeptide analysis of calf thymus histone H1 phosphorylated *in vitro* by Pim-1 kinase resulted in fragments containing sequences similar to that of the preferred synthetic substrate peptide shown above. Therefore, under optimized *in vitro* conditions, the substrate recognition sequence for Pim-1 kinase is (Arg/Lys)3-X-Ser/Thr*-X', where X' is likely neither a basic nor a large hydrophobic residue. This work was funded in part by NIH grant AI-26356 and USDA grant 91-37206-6867.

BZ 346RAPID PHOSPHORYLATION OF THE P42/P44 MAP KINASES AND RAF-1 IN RESPONSE TO ESTROGEN IN 3T3 CELLS EXPRESSING A RAF PROTEIN KINASE:ESTROGEN RECEPTOR HORMONE BINDING DOMAIN FUSION PROTEIN. Martin McMahon. DNAX Research Institute, 901 California Ave, Palo Alto, California 94304.

Fusion of the hormone binding domain of the estrogen receptor (hER) to the carboxy-terminus of a variety of constitutive protein kinase oncogenes renders transformation by these molecules dependent upon the addition of exogenous estradiol. We have used this observation to generate a conditional allele of the human raf protein kinase (hrafER) and are employing this conditionality to investigate the phosphorylation of substrates downstream of raf in signal transduction pathways. Activation of the hrafER molecule by the addition of exogenous estradiol or hydroxy-tamoxifen leads to the phosphorylation of the p42/p44 MAP kinases and the endogenous $p74^{raf-1}$ as assessed by mobility shift in a polyacrylamide gel. This phosphorylation occurs within 5 minutes after the addition of estradiol and is maintained in the continuous presence of estradiol. Pre-treatment of cells with the protein synthesis inhibitor cycloheximide followed by the addition of estradiol leads to a greater proportion of both p42 and p44 shifting to a higher molecular weight. Indeed under these conditions 50% of p42 and 100% of p44 show a mobility shift. Anti-phosphotyrosine western blotting indicates that p42 becomes tyrosine phosphorylated which would suggest that the phosphorylation of p42 is dependent upon the recently described MAP kinase kinase. These results indicate that the raf protein kinase is upstream of the p42/44 MAP protein kinases and suggests that raf may be an activator of MAP kinase kinase. The phosphorylation of the endogenous c-raf-1 may be due to the direct action of the hrafER protein or may be due to the activation of the MAP kinases as has been suggested by others. We are presently investigating the consequences of phosphorylation of p42/p44 and c-raf-1 on the kinase activity of these molecules and on subsequent gene activation. In addition we are continuing to use this conditional allele to look for other candidate substrates of the raf protein kinase.

BZ 347 EFFECTS OF PHORBOL ESTER ON MAP KINASE ACTIVATOR IN WILD-TYPE AND PHORBOL ESTER-RESISTANT EL4 THYMOMA CELLS, Kathryn E. Meier and Katrina C.Gause, Department of Pharmacology, Medical University of South Carolina, Charleston SC 29425.

MAP kinase (MAPK) is a serine/threonine kinase that is activated in response to treatment of mammalian cells with growth factors and mitogens. MAPK is activated when phosphorylated by MAPK activator. a MAPK kinase. In wild-type (WT) EL4 murine thymoma cells, addition of phorbol ester (PMA) results in activation of protein kinase C (PKC) and in transcription of interleukin-2 (IL-2). A phorbol-ester resistant variant of the EL4 cell line does not transcribe IL-2 in response to PMA. We have shown that WT but not variant EL4 cells activate MAPK when treated with PMA. However, both WT and variant cells express activatable PKC and MAPK. The goal of this study was to examine the production of MAPK activator in WT and variant EL4 cells. Cytosolic extracts from control and PMA-treated EL4 cells were fractionated by Mono Q chromatography. Activator activity was assessed by a method in which column fractions were first incubated in the presence of ATP for 15 min at 30°. Myelin basic protein (MBP), a MAPK substrate, was then added for an additional 15 min. When both activator and MAPK were present in the incubation, enhanced phosphorylation of MBP was detected. Activator from WT cells eluted from Mono Q at a concentration of 50 mM NaCl when cytosolic extracts of PMA-treated cells were partially purified on Mono Q. A portion of the inactive MAPK co-eluted with the activator, such that MAPK activation could be detected by incubating this fraction alone. Activator was detected only in PMA-treated WT cells, and not in PMA-treated variant cells. To determine whether the MAPK from variant cells was responsive to activator, fractions containing the major peak of inactive MAPK (200-250 mM NaCl) were used as the source of MAPK for the activator assay. MAPK from variant cells could be activated by activator from WT cells. However, little MAPK activator activity could be detected in PMA-treated variant cells using MAPK from either WT or variant cells. These results indicate that variant EL4 cells do not produce active MAPK activator in response to PMA. Thus, the signalling defect in the phorbol ester-resistant cells is localized to a step between PKC and MAPK activator.

BZ 348 RAF-1 MEDIATES GROWTH-RELATED GENE EXPRESSION.

R. J. Miltenberger and P. J. Farnham, McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin.

The proto-oncogene Raf-1 is a ubiquitously expressed cytoplasmic serine/threonine kinase believed to be one of the key molecules which transmit external mitogenic stimuli to the nucleus. The oncogenic form of Raf-1, v-Raf, is a constitutively activated Raf kinase that has been shown to stimulate transcription from the immediate early growth response genes *c-fos*, *β-actin*, and *egr-1*. To investigate the role of Raf-1 in mediating growth-related gene expression later in the cell cycle, we have monitored the effect of different mutant Raf-1 expression constructs on the transcriptional activity of a battery of early and late serum-responsive genes via transient cotransfection of NIH3T3 cells with luciferase reporters. v-Raf strongly transactivates the immediate early growth response genes *c-fos* and *egr-2*, the mid-G_1 response gene ODC, and the late growth response gene *Rep-3*. Two other late G_1/S phase specific genes, CAD and DHFR, are only mildly transactivated by v-Raf, however. When quiescent cells are serum stimulated to re-enter the proliferative cell cycle in the presence of the inhibitory Raf-1 mutants RafC4 or Raf 301, transcription of *egr-2*, but not *c-fos*, is significantly suppressed. Therefore, Raf-1 may play a greater role in regulating *egr-2* gene expression than it does *c-fos*. Other growth-related genes which are highly transactivated by v-Raf are being tested for suppression by RafC4 and Raf301 to determine if endogenous Raf-1 is necessary for their serum-induced expression throughout the proliferative cell cycle. Novel Raf-1-responsive promoter elements are also being mapped and characterized.

BZ 349 **INTERACTION OF A SOLUBLE MAP KINASE COMPLEX WITH p21$^{c\text{-}Ha\text{-}ras}$.GTP AND THE ACTIVATED Val12 Ha-ras MUTANT.**

Shonna Moodie, Berthe Willumsen[1] and Alan Wolfman, Department of Cell Biology, Cleveland Clinic Foundation, 9500 Euclid Av., Cleveland, OH 44106 and [1]Department of Microbiology, University of Copenhagen, Copenhagen, Denmark.

We have detected a 42Kda protein associating specifically with immobilized -p21$^{c\text{-}Ha\text{-}ras}$.GMP-PNP, which in the presence of γ^{32}P-ATP becomes heavily phosphorylated. The phosphorylation of the p42 is not detected when crude rat brain lysates are incubated with immobilized-p21$^{c\text{-}Ha\text{-}ras}$.GDP. Following phosphorylation, a portion of the p42 is specifically released from the immobilized-p21$^{c\text{-}Ha\text{-}ras}$.GMP-PNP. Immunoprecipation of these ATP eluted supernatants with anti p42^{mapk} results in the detection of phosphorylated MAP kinase only in the presence of p21$^{c\text{-}Ha\text{-}ras}$.GMP-PNP. These observations were confirmed by the detection of MAP kinase activity (phosphorylation of MBP without activity directed towards either casein or histone) in immobilized-p21$^{c\text{-}Ha\text{-}ras}$.GMP-PNP pellets and its ATP eluted supernatants. Clearing the ATP eluted supernatants with anti p42^{mapk} removed all MAP kinase activity. This supernatant, however, retained the ability to activate recombinant p42^{mapk}, indicating the presence of a MAP kinase activator. We have observed the presence, though to varying degrees, of both the MAP kinase and its activator associating with both the GDP and GTP forms of the oncogenic Val12 mutant.This suggests that the association between p21$^{c\text{-}Ha\text{-}ras}$.GMP-PNP and a MAP kinase complex might play a significant role in the generation of a ras-dependent proliferative signal. Under identical conditions, and supportive of this hypothesis, we did not detect the association of the MAP kinase complex with the Ala36 effector domain mutant.

We would like to thank Michael Weber for providing us the anti p42^{mapk} antibodies and recombinant p42^{mapk}.

BZ 350 ACTIVATION OF IMMEDIATE EARLY GENES IN RESPONSE TO ERK1 GENE EXPRESSION

Sun-Yu Ng and Chiou-Fen Chuang, Institute of Molecular Biology, Academia Sinica, NanKang, Taipei 11529, Taiwan, Republic of China

The activation of immediate early genes by growth factors is believed to involve cytoplasmic protein-serine/threonine kinases. In order to identify the cytoplasmic kinases which are responsible for the transduction of mitogenic signals into the nucleus, we have co-transfected a reporter gene under the control of the human β-actin promoter together with ERK1 or ERK2 expression plasmid. We found that both CAT enzyme activity and CAT mRNA level were induced in the presence of an ERK1 expression vector. In order to identify other targets of the MAP kinase encoded by ERK1, this cDNA was put under the control of an inducible metallo-thionein promoter. In stable transfectants of this ERK1 expression plasmid, ERK1 and β-actin mRNA levels were both induced by cadmium chloride. Besides activating the β-actin gene, several cellular genes also were induced and other genes appeared to be repressed. Since MAP kinase activity correlates with tyrosine phosphorylation, an anti-phosphotyrosine antibody was used to detect autophosphorylated MAP kinase, whose level was found to increase in response to cadmium chloride.

BZ 351 **SELECTIVE ACTIVATION OF NOVEL PROTEIN KINASE C MEMBERS UPON SERUM STIMULATION OF QUIESCENT RAT 3Y1 FIBROBLASTS.**

Shigeo Ohno, Keiko Mizuno, Shu-ichi Hirai, Shin-ichi Osada and Akiko Hata, Department of Molecular Biology, Yokohama City University School of Medicine, 3-9, Fuku-ura, Kanazawa-ku, Yokohama 236, Japan

Protein kinase C (PKC) has been implicated in a variety of cell functions which are mediated by diacylglycerols and mimicked by tumor-promoting phorbol esters. PKC constitutes a protein family which could be divided into three distinct classes. Ca-sensitive, conventional PKC contains α, βI, βII, and γ. Ca-insensitive, novel PKC members contains δ, ϵ, η, θ, ζ, and λ. *All the members except* ζ and λ show kinase activity regulated by diacylglycerols or phorbol esters and also bind phorbol dibutylate. Thus they are the candidates for the physiological targets of diacylglycerol and the phorbol esters. The presence of multiple PKC members with distinct structural and biochemical properties suggests functional diversity of the members. However, there has been few demonstration of the involvement of a specific PKC member in a specific cell function. To elucidate the functional differences among the PKC members in intact cells, we employed a transient assay system where overexpression of a specific PKC member results in an enhancement of a cell response to TPA. The cell response were measured in terms of transcriptional activation of a reporter CAT gene which contains TPA-response elements(TRE) upstream of the CAT gene. Overexpression of various PKC members do not alters the basal CAT expression. However, overexpression of PKC α, β, δ, ϵ, and η results in the enhancement of CAT expression in response to TPA, suggesting that all these members respond to TPA and mediate the signal to TRE. By using this system as an assay to monitor the activation levels of respective PKC members, we next examined which PKC members in quiescent rat 3Y1 fibroblasts are activated by serum stimulation. *Quite interestingly, overexpression of the nPKC members but not* cPKC members resulted in the great enhancement of the CAT expression in response to serum, although similar enhancements were obtained in response to TPA. The results suggest that serum activation of quiescent fibroblasts resulted in a selective activation of nPKC members. This implies the specific involvement of the nPKC member (δ and ϵ in 3Y1 cells) in serum activation of the quiescent cells. Further studies to identify the substance in the serum which cause the selective activation of nPKC members are now in progress.

BZ 352 THE STIMULATION OF PP42MAPKINASE BY INSULIN DOES NOT CORRELATE WITH ITS METABOLIC ACTIONS IN CELLS OVEREXPRESSING MUTANT INSULIN RECEPTORS. Long Pang§†, David E. Moller‡, Jeffrey S. Flier‡, and Alan R. Saltiel§†, §Department of Physiology, University of Michigan, School of Medicine, Ann Arbor, MI 48109. †Department of Signal Transduction, Parke-Davis Pharmaceutical Division, Ann Arbor, MI 48105. ‡The Charles A Dana Research Institute and Harvard-Thorndike Laboratory of Beth Israel Hospital, Department of Medicine, Beth Israel Hospital and the Harvard Medical School, Boston, MA 02215

Naturally occurring human insulin receptor mutants Ser^{1200} and Thr^{1134}, and a site-directed mutant Arg^{1030} overexpressed in Chinese hamster ovary (CHO) cells bind insulin with affinities identical to wildtype receptors but are apparently kinase deficient. However, cells expressing the Ser^{1200} receptor exhibit insulin stimulation of glucose transport and glycogen synthesis similar to wildtype receptor, but fail to mediate insulin-responsive DNA synthesis. In contrast, the Thr^{1134} and Arg^{1030} mutants exhibit no response to insulin. The activity of Mitogen Activated Protein (MAP) kinase in cells transfected with wildtype receptor is more responsive to insulin then untransfected parental cells, while cells bearing any of the mutant receptors are less responsive to insulin. These differences in the stimulation of MAP kinase are paralleled by differences in insulin-dependent phosphorylation of the enzyme. Insulin stimulates the tyrosine phosphorylation of the 42 kd MAP kinase in cells expressing wildtype receptors but not any of the mutants, as detected by immunoblotting. Phosphorylation of the immunoprecipitated MAP kinase is also examined by [^{32}P]orthophosphate labeling. Insulin markedly stimulates the phosphorylation of the protein in cells expressing wildtype receptors. Only trace phosphorylation is observed in the mutant cell lines, although phosphotyrosine, phosphoserine and phosphothreonine are all detected. These results suggest that the p42 MAP kinase is not universally required for the metabolic effects of insulin.

BZ 353 nPKCμ A NOVEL, MEMBRANE BOUND PROTEIN KINASE OF THE PKC FAMILY. K. Pfizenmaier, F.J. Johannes, S. Eis, Institute of Cell Biology and Immunology, University of Stuttgart, 7000 Stuttgart 80, FR GERMANY

In order to identify new protein kinase genes which are possibly involved in cytokine receptor mediated signal transduction, we have isolated and characterized a new gene coding for a putative Ser/Thr specific protein kinase. Using degenerate oligonucleotides from conserved regions of the kinase domain and PCR amplification of positive cDNA fragments, a full length 3.7 kb cDNA from a human placenta library could be isolated. This cDNA codes for an open reading frame of 912 amino acids. Translation of in vitro synthesized T7 transcripts from this cDNA in a rabbit reticulocyte lysate results in a predominant protein band of 115 kD.

Sequence comparison of the deduced amino acids to several data bases showed homologies to the PKC-family, with best fit to the nPKC subtypes, which lack the Ca^{++} binding domain C2. The similarities are restricted to two characteristic conserved domains, the duplicated cysteine rich "zinc finger like structures" (C1), responsible for Phorbolester binding, and the catalytic protein kinase domain (C3). More interestingly, unique structural features of the new gene reside in the long, N-terminal D1 region, which starts with a typical leader sequence and cleavage signal, followed by a putative transmembrane domain, as predicted from hydropathy plot. Such a structure has so far not been described for other members of the PKC family and suggest an involvement of this kinase in certain signalling pathways, potentially via direct linkage to nonkinase membrane receptors.

To determine the expression of this protein kinase gene, ^{32}P-labelled cDNA probes were used to screen total cellular RNAs of human cell lines and tissues of different origin. Northern hybridisation analyses revealed low expression of a single 3.7 kb transcript in placenta and strong expression in some carcinoma cell lines. The gene is located on a 5 kb and 7 kb Eco RI chromosomal fragment as a single copy, containing at least one intron with an internal Eco RI site as indicated from Southern-blot analysis. Further, we have deteted homologous genes in yeast and murine DNA.

Based on the homologies to the characteristic domains of PKCs and considering the putative TM domain, we have provisionally termed this novel kinase nPKCμ for novel, membrane bound protein kinase C.

BZ 354 PHOSPHORYLATION OF GLYOXYSOMAL MALATE SYNTHASE EFFECTS OLIGOMERIZATION, Douglas D. Randall, John Finnessy and Yan-Ping Yang, Biochemistry Department, University of Missouri, Columbia, MO 65211

Malate synthase catalyzes one of the two unique reactions of the glyoxylate cycle that allows oil rich seeds to convert up to 70% of storage lipids to carbohydrates. Malate synthase (MS) is found in the monomer, dimer and oligomeric forms, with the oligomeric exhibiting the greatest activity. In Ricinus communis L. endosperm tissue, the monomeric form (5S form) is apparantly phosphorylated prior to oligomerization into the 20S form by a glyoxysomal malate synthase kinase. MS is phosphorylated on one serine residue per 64kD subunit of MS. In vitro phosphorylation of 5S MS is inhibited by EGTA, fluoride and molybdate suggesting that the kinase is calcium activated and may also require phosphatase action to activate the kinase. Dephosphorylation of the 20S MS by alkaline phosphatase results in dissociation of the oligomeric MS to its monomeric form and loss of greater than 90% of its activity. Dephosphorylated MS is a poor substrate for cAMP protein kinase and is not phosphorylated by either of two calcium dependent protein kinases that we have purified from endosperm tissue. In order to obtain large quantities of MS, we are overexpressing the MS clone in E. coli using the pGEX-2T vector. Ongoing studies include isolation of the MS protein kinase, and identification of the phosphorylation site in MS. To our knowledge phosphorylation of glyoxysomal malate synthase is the only known enzyme to be phosphorylated in peroxisomes and implies the presence of a novel peroxisomal protein kinase.

BZ 355 SUBUNIT COMPOSITION OF CASEIN KINASE II FROM S. CEREVISIAE: EVIDENCE THAT THE YEAST ENZYME CONTAINS TWO DISTINCT REGULATORY BETA SUBUNITS. Ashok P. Bidwai, J. Craig Reed and Claiborne V.C. Glover, Department of Biochemistry, The University of Georgia, Athens, GA 30602

Casein kinase II (CKII) is a multifunctional Ser/Thr protein kinase ubiquitous among eukaryotes. The enzyme from yeast contains two distinct catalytic subunits, α (42kDa) and α' (35kDa), and two putative regulatory β subunits of 41 and 32kDa (Padmanabha et al., 1990, Mol. Cell. Biol. 10:4089-4099). Antibodies raised against the β subunit of Drosophila CKII crossreact with the 41kDa subunit but not with the 32kDa polypeptide. Furthermore, antibodies raised against the 41kDa subunit do not crossreact with the 32kDa polypeptide and vice versa, indicating a lack of precursor-product relationship. Thus, the 32kDa polypeptide may represent either a divergent β subunit, a contaminant, or a third subunit unique to yeast.

In order to define the biological role of the β subunit(s) the precise subunit composition of the enzyme with respect to the β subunit(s) must be known. We report here the results of sequencing studies designed to confirm the identity of the putative yeast β subunits. Yeast CKII was isolated by a modified purification procedure that provides a 5-fold higher yield of homogenous enzyme with a subunit composition similar to that described earlier. The 41 and 32kDa polypeptides were isolated by preparative SDS polyacrylamide gel electrophoresis and cleaved using trypsin or V-8 protease. The peptides were separated by reverse phase HPLC and sequenced by automated Edman degradation. Both the 41 and 32kDa subunit-derived peptides gave sequences with homology to β subunit sequences of other organisms, confirming that both the 41 and 32kDa polypeptides are β subunits. When combined with the immunological results, these data indicate that yeast CKII contains two distinct regulatory subunits. This represents the first demonstration of β subunit heterogeneity in CKII from any source.

Supported by grants NIH GM33237 and ACS MV-463.

BZ 356 PROTEIN KINASE (PK)A AND PKC PATHWAYS INDEPENDENTLY REGULATE P450-CHOLESTEROL SIDE CHAIN CLEAVAGE mRNA LEVELS IN Y1 ADRENAL CELLS, Mary E. Reyland, Department of Pharmacological Sciences, SUNY, Stony Brook, NY 11794.

Steroidogenesis in adrenocortical cells is stimulated by activators of PKA, and suppressed by activators of PKC. To investigate the relationship between these pathways, I have utilized the mouse Y1 adrenal cell line. dBtcAMP increases steroid production in Y1 cells up 10-fold. Stimulation of steroidogenesis is bi-modal; the 1st phase begins within 30 mins, followed by a 2nd phase at about 4 hours. Staurosporine and calphostin C, two potent inhibitors of PKC, likewise increase steroidogenesis in Y1 cells 3-5 fold. Stimulation of steroidogenesis by inhibitors of PKC requires 3-4 hr and corresponds to the 2nd phase of stimulation by dBtcAMP. Northern blot analysis demonstrates that treatment with either dBtcAMP, or inhibitors of PKC, increases expression of P450-cholesterol side chain cleavage (SCC) mRNA, the rate limiting enzyme in steroid synthesis, up to 5 fold. SCC mRNA expression increases in parallel with stimulation of steroidogenesis by the PKC inhibitors. To determine if stimulation of steroidogenesis by PKC requires PKA, Y1 cells were treated with staurosporine in the presence of Rp-8-Br-cAMP, a specific inhibitor of PKA. Rp-8-Br-cAMP had no effect on stimulation of steroidogenesis by staurosporine, while dBtcAMP-stimulated steroidogenesis was inhibited >80%. To determine if regulation of SCC mRNA expression by PKC requires PKA, I utilized the PKA deficient Y1 kin 8- cell line. Northern blot analysis shows that induction of SCC mRNA expression by calphostin C or staurosporine is comparable in Y1 and Y1 kin 8- cells. These data indicate that PKA and PKC regulate steroidogenesis through independent effects on SCC mRNA expression.

BZ 357 THE ROLE OF MAP KINASES IN GLUCOSE TRANSPORTER TRANSLOCATION IN 3T3-L1 ADIPOCYTES, Linda J. Robinson, Zuhaira F. Razzack, and David E. James, Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63130.

Insulin regulates glucose transport in fat and muscle cells by translocating a glucose transporter (GLUT4) from within the cell to the plasma membrane. We have previously shown that okadaic acid, an inhibitor of type I and IIa phosphatases, and GTPγS, both stimulate GLUT4 translocation in adipocytes. In the present studies, we have shown that another phosphatase inhibitor, calyculin A, also stimulates GLUT4 translocation in 3T3-L1 adipocytes. Each of these agents stimulated phosphorylation of 2 MAP kinase isoforms of Mr 42 and 44 kD, consistent with the observed Mr of pp42mapk/erk2 and pp44mapk/erk1/mpk. Phosphorylation was determined by immunoblotting 3T3-L1 cell extracts with an anti-phosphotyrosine antibody (kindly provided by Thomas Roberts) or an anti-MAP kinase antibody (kindly provided by John Lawrence). In contrast to the phosphatase inhibitors, potassium fluoride (KF) stimulated phosphorylation of MAP kinases, but did not promote GLUT4 translocation. Insulin-induced translocation of GLUT4 was not inhibited by KF, however, indicating that KF did not inhibit downstream effectors involved in GLUT4 translocation. Conversely, dibutyryl cAMP stimulated GLUT4 translocation without causing MAP kinase phosphorylation. Collectively these data suggest that MAP kinase may not be an essential regulator of glucose transport in insulin-sensitive tissues.

BZ 358 A NOVEL PROTEIN KINASE GENE IN *ARABIDOPSIS*, Judith L. Roe, *Carol Rivin, Allen Sessions, ‡Kenneth Feldmann and Patricia C. Zambryski, Dept. of Plant Biology, University of California, Berkeley, CA 94720, Dept. of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331, ‡Dept. of Plant Sciences, University of Arizona, Tucson, AZ 85721

The *tousled* mutation of *Arabidopsis thaliana* exhibits defects in pattern formation during flower development. Flowers of homozygous *tsl* plants each contain a random set of floral organs (sepals, petals, stamen and a bicarpellate pistil); their position is correct, but their number is reduced. Light and electron microscopy indicates that the *TSL* gene acts early during flower development. Leaves of the mutant are also slightly abnormal, displaying deeper serrations on their margins than wild-type leaves. A *tsl* mutant line has been generated which contains a T-DNA insertion element that segregates with the mutant phenotype. The T-DNA has been inserted into a gene which encodes a novel plant protein kinase (PK). The wild-type PK gene is currently being transformed into mutant plants to confirm that the gene can rescue the mutant phenotype (and is therefore the *TSL* gene). The gene encodes a 78 kDa protein with three structural domains. The C-terminal half of the protein shares the conserved residues found in the catalytic domain of known PKs, and is most homologous to the serine/threonine class of PKs. It does not fall into any known subgrouping of PKs, however, and may represent a new class of PKs. The N-terminal half of the protein consists of two sub-domains. A glutamine-rich domain is followed by an α-helical domain which shows some homology to the rod domain of myosin heavy chains and the α-helical region of intermediate filaments. Several nuclear-localization signals are found in the N-terminal domain. The protein does not appear to be a trans-membrane receptor, as no membrane-spanning regions can be identified. To determine when and where this PK gene may be functioning during normal development in the plant, Northern blot analysis was performed. The PK gene is most abundantly expressed in flowers, but the transcript can also be detected (at lower levels) in both roots and leaves. Ultrastructural studies reveal that individual cells in *tsl* organs are abnormal in appearance, suggesting that the *TSL* gene product may participate in a pathway which affects cellular structure (and subsequently pattern formation) during normal plant development.

BZ 359 PROTEIN KINASE C ISOZYMES PROLONG ACTIVATION AND TYROSINE PHOSPHORYLATION OF EXTRACELLULAR SIGNAL-REGULATED KINASES, Reina Roivainen and Robert O. Messing, Department of Neurology, Gallo Center, University of California, San Francisco, CA 94110

Extracellular signal-regulated kinases (ERKs) are activated by phosphorylation on threonine and tyrosine in response to certain mitogens and growth factors. In PC12 cells, nerve growth factor (NGF) activates ERKs by a process that is partly dependent on protein kinase C (PKC). We have examined the mechanisms and PKC isozymes involved in this process. Treatment of PC12 cells with NGF (50ng/ml) for 2.5 - 60 min increased ERK activity, assayed as phosphotransferase activity towards the peptide APRTPGGRR (Clark-Lewis, et al., J. Biol. Chem. (1991) 266:15180-15184). The increase was maximal (4-fold) at 5 min, decreased to 2-fold within 10 min and persisted at this level up to 60 min. NGF also induced phosphotyrosine immunoreactivity (PTYR-IR) of 42- and 45 kDa proteins identified as ERKs by immunoprecipitation with an anti-ERK-antibody. Induction of PTYR-IR followed a similar time course as the increase in ERK activity. Treatment with 1 μM phorbol 12-myristate 13-acetate (PMA) for 24 hours depleted PC12 cells of PKCα, β, δ and ε; our clone does not contain PKCγ. In PKC-depleted cells, the response to NGF was reduced slightly (10-20%) after 5 min, but at 10-60 mins of NGF exposure, ERK activity and phosphorylation were reduced by 30-40 %. Simultaneous treatment with NGF and 10 nM PMA, which activates PKC, resulted in persistence of ERK activity and PTYR-IR at maximal levels up to 60 min. A similar effect was seen with NGF and 10 nM thymeleatoxin, a phorbol ester that selectively activates PKCα, β and γ, but not δ or ε [Ryves et al., FEBS Lett (1991) 288:5-9]. Activation of PKC by 10 nM PMA alone induced only minor activation and PTYR-IR of ERKs. The results suggest that PKCα or β potentiate and prolong NGF-induced ERK phosphorylation and activation. Experiments are in progress to determine whether enhancement of ERK activity by PKC is due to inhibition of a tyrosine phosphatase.

BZ 360 TYROSINE PHOSPHORYLATION OF A 42 kDa ERK2 KINASE IS ASSOCIATED WITH MAXIMAL IL-2 PRODUCTION, Lijun Song, William H. Adler, Susie Chung, Young Ho Kim, Gary D. Collins and James E. Nagel, Clinical Immunology Section, GRC/NIA/NIH, Baltimore, MD 21224

Although tyrosine phosphorylation of certain cellular proteins is an obligatory event for IL-2 secretion, it is not known which tyrosine phosphorylated protein(s) is responsible for IL-2 production. In this study, the relationship between IL-2 production and the protein tyrosine phosphorylation pattern of human Jurkat T cells was investigated using phosphotyrosine immunoblotting analysis. With anti-CD3 or anti-CD2 activation the cells showed only a low (anti-CD3) or a moderate (anti-CD2) level of tyrosine phosphorylation of a 42 kDa protein, which was accompanied by undetectable (anti-CD3) or low level (anti-CD2) IL-2 production. In the presence of 10 ng/ml of PMA, large amounts of IL-2 were induced by both anti-CD3 and anti-CD2 stimulation and this was accompanied by high levels of tyrosine phosphorylation of the 42 kDa protein. Anti-ERK immunoblotting and in vitro ERK kinase assays, using myelin basic protein as a substrate, demonstrated that the 42 kDa protein is a member of the ERK (external signal regulated kinase) serine/threonine kinase family and that the kinase activity of this ERK kinase paralleled its phosphotyrosine content. These observations suggest that tyrosine phosphorylation and activation of the 42 kDa ERK kinase is involved in and necessary for IL-2 production. In addition, tyrosine phosphorylation of a 100 kDa protein also seems to be involved in IL-2 production since PMA alone, which induced high levels of tyrosine phosphorylation of the 42 kDa ERK, neither induced any detectable IL-2 nor increased the level of tyrosine phosphorylation of the 100 kDa protein, both of which were significantly induced by both anti-CD3 or anti-CD2 in the presence of PMA.

BZ 361 ACTIVATION OF THE raf-1 PROTEIN KINASE BY PROTEIN KINASE C PHOSPHORYLATION. Silvia Stabel, Osman Sözeri, Marek Liyanage, Betina Marquardt and George E. Mark III*, Max-Delbrück-Laboratorium in der Max-Planck-Gesellschaft, Carl-von-Linné-Weg 10, D-5000 Köln 30, Germany, *Merck Sharp & Dohme Research Laboratories, Rahway, USA

We have constructed recombinant baculoviruses expressing the wild-type c-raf protein, a kinase-deficient mutant and a mutant lacking the zinc finger region of c-raf. When expressed in insect cells the wild-type protein shows very low kinase activity upon extraction and immunoprecipitation, the kinase-negative mutant is unable to phosphorylate.

However, if c-raf is co-expressed in insect cells with mammalian conventional protein kinase C (PKC) members (PKC-α, PKC-β1, PKC-β2, PKC-γ) the kinase activity of the Raf protein *in vitro* is strongly stimulated. Using a kinase-deficient PKC mutant we show that the activating effect of PKC depends on the presence of a functional kinase domain in this molecule. In addition, PKC only exerts its activating effect on the c-raf kinase in the presence of the phorbol ester TPA/PMA (1).

The activation of c-Raf by PKC appears to be due to a direct phosphorylation of the c-Raf protein by PKC since *in vitro* phosphorylation of the Raf protein with purified PKC also leads to activation of the kinase activity.

The novel PKC members (PKC-δ, PKC-ζ, PKC-η) are not able to activate the Raf kinase in a TPA-dependent manner.

1) Sözeri, O.; Vollmer, K.; Liyanage, M.; Frith, D.; Kour, G.; Mark, G.E. III and Stabel, S. Oncogene (in press)

BZ 362 RAPAMYCIN INHIBITS RIBOSOMAL S6 PHOSPHORYLATION, DECREASES PROTEIN SYNTHESIS & CELL SIZE, BUT NOT CELL PROLIFERATION IN CYCLING CELLS. **Naohiro Terada, Kozo Takase, Joseph J. Lucas and Erwin W. Gelfand.** Department of Pediatrics, Division of Basic Sciences, National Jewish Center for Immunology and Respiratory Medicine, Denver, CO 80206.

Rapamycin has been demonstrated to selectively and universally inhibit phosphorylation and activation of p70 S6 kinase in all cell types. In continuously growing Ramos cells, a B-cell line derived from Burkitt lymphoma, rapamycin also induced the dephosphorylation and inactivation of p70 S6 kinase within 1-3 hours. In parallel to these events, phosphorylation of ribosomal S6 protein was completely inhibited, protein synthesis was partially (20-40% of control) inhibited, and the cell size became significantly smaller. However, of interest, the cells continue to stay in cell cycle, and divide with an apparently normal DNA synthesis at least 3 more cycles after addition of the drug. Rapamycin also exerted a minimal effect on cell proliferation of exponentially growing other cell lines. In contrast, DNA synthesis was markedly inhibited by rapamycin when these cell lines were growth arrested in resting status and then stimulated with serum or mitogens. These findings suggest that inhibition of S6 phophorylation by rapamycin may be critical for (re-)entry into cell cycle from a resting status, but not for the regulation of DNA synthesis in cycling cells.

BZ 363EPIDERMAL GROWTH FACTOR STIMULATES PHOSPHORYLATION OF EUKARYOTIC INITIATION FACTOR 4B, INDEPENDENTLY OF PROTEIN KINASE C,

Harry O. Voorma, Rob M.F. Wolthuis, and Johannes Boonstra, Department of Molecular Cell Biology, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands.

Cell division, especially of quiescent cells , requires in addition to DNA synthesis, also enhanced rates of RNA- and protein synthesis, and it seems attractive to assume that these latter processes are also under direct control of the receptor kinase in order to realize the required specific adaptation. For protein synthesis these targets are most likely eukaryotic initiation factors, involved in the messenger binding to the 40S ribosomal subunit, since regulation is exerted at this level of initation.

Highly phosphorylated forms of eIF-4B are observed within one minute of exposure of human A431 epidermoid carcinoma cells to EGF. It suggests that eIF-4B phosphorylation is caused by a kinase which is active early-on in the EGF-induced signal transduction cascade, although the lack of phosphotyrosine residues excludes direct phosphorylation by the activated EGF-receptor kinase. Addition of PMA, an activator of PKC, also results in highly phosphorylated forms of eIF-4B. However, PKC is not the only mediator in the EGF-induced phosphorylation, since the EGF effect was unimpaired in cells that are down-regulated for PKC activity. An indication as to the nature of the unknown kinase involved, is obtained when the Ca^{2+}-ionophore A23187 appears also capable to induce a similar pattern of phosphorylated isomers of eIF-4B and suggests that eIF-4B phosphorylation is a Ca^{2+} -dependent process. The observation that eIF-4B is phosphorylated rapidly after addition of growth factors suggests that growth factor-induced signal transduction results in rapid regulation of major cellular processes, one of which may be the stimulation of selective translation of stored mRNAs by means of unwinding the 5' untranslated regions of these mRNAs. It is known that phosphorylated eIF-4B displays an enhanced unwinding activity towards these 5' UTRs.

BZ 364 IDENTIFICATION OF A MITOGEN-STIMULATED PHOSPHORYLATION SITE WITHIN THE N-TERMINAL REGULATORY DOMAIN OF RAF-1. Markus Wartmann¶, Howard M. Price#, Victor Lazaron¶, and Roger J. Davis*¶, Howard Hughes Medical Institute*, Program in Molecular Medicine¶, the Department of Biochemistry and Molecular Biology¶, and the Division of Urology, Department of Surgery#, University of Massachusetts Medical School.

Raf-1, the protein product of the cellular homolog of the v-*raf* oncogene, is a ubiquitously expressed serine/threonine protein kinase which is activated in response to a wide array of mitogenic stimuli. Treatment of Chinese hamster ovary cells with serum or phorbol ester elicited responses characteristic for Raf-1 activation: i) a mobility-shift upon SDS-PAGE, an increase in ii) the *in vivo* phosphorylation state and iii) the *in vitro* autophosphorylation, of Raf-1. A serine residue within the N-terminal regulatory domain of Raf-1 was identified as an *in vivo* site of Raf-1 phosphorylation by site-directed mutagenesis and phosphopeptide mapping. Significantly, a Raf-1 mutant, in which this serine residue had been replaced with Ala, was partially impaired in all three mitogen-stimulated responses described above. Taken together, these data demonstrate that phosphorylation at this site contributes, in part, to the mechanism of activation of Raf-1. The incomplete block in Raf-1 activation caused by the mutation at this site, however, indicates that the mechanism of activation may require concerted phosphorylation of Raf-1 at more than one site. A potential Raf-1 kinase kinase candidate is ERK2, since this MAP kinase isoform phosphorylated this site *in vitro*. However, phosphorylation of Raf-1 by MAP kinase was insufficient to cause changes in Raf-1 properties characteristic for its activation. This implies that phosphorylation of Raf-1 by ERK2, or a kinase with a related substrate specificity, may be necessary, but is not sufficient, to account for Raf-1 activation. Further experiments are required to determine the identity of the kinases responsible for the activation of Raf-1 *in vivo*.

BZ 365 LPS-INDUCED PROTEIN TYROSINE PHOSPHORYLATION IN MACROPHAGES IS MEDIATED BY CD14 AND LEADS TO THE ACTIVATION OF MAP KINASES, Steven L. Weinstein, Carl H. June, Jasbinder S. Sanghera, Steven L. Pelech and Anthony L. DeFranco, Departments of Physiology and Microbiology/Immunology, University of California, San Francisco, CA 94143, Naval Medical Research Institute, Bethesda, MD 20814 and the Biomedical Research Center, University of British Columbia, Vancouver, BC V6T1Z3 Canada.

Bacterial lipopolysaccharide (LPS) is a potent activator of responses by macrophages that modulate the host defense against bacterial infection. An early consequence of LPS stimulation is increased protein tyrosine phosphorylation, an event that appears to mediate some macrophage responses to LPS. To better understand the role of induced tyrosine phosphorylation in LPS-stimulated macrophages, we attempted to identify some of the components that participate in this response. We found that the putative LPS receptor, CD14, mediated LPS-induced tyrosine phosphorylation. Antibodies to CD14 that prevent the binding of LPS to CD14, specificially inhibited the induction of protein tyrosine phosphorylation stimulated by LPS concentrations below 1 ng/ml. At higher LPS doses, however, anti-CD14 antibodies did not block the tyrosine phosphorylation response, suggesting that a CD14- independent mechanism mediates the induction of protein tyrosine phosphorylation at LPS concentrations greater than 1 ng/ml. These results are concordant with data reported for the CD14 dependence of LPS-stimulated functional responses in macrophages. Thus, induced protein tyrosine phosphorylation is coupled to the CD14-dependent and -independent mechanisms for LPS recognition that are thought to mediate the biological responses of macrophages to LPS. In addition, we found that two of the prominently induced, tyrosine phosphoproteins (41-kD and 44-kD) in LPS-stimulated macrophages correspond to isoforms of MAP kinase. Each of these proteins was specifically immunoprecipitated by anti-MAP kinase antibodies and both proteins co-migrated with MAP kinase activity in MonoQ anion-exchange column fractions. Moreover, LPS treatment produced a large increase in MAP kinase activity. Inhibition of LPS-induced tyrosine phosphorylation of these two proteins was accompanied by a loss of elevated MAP kinase activity. These results suggest that LPS induces the tyrosine phosphorylation of at least two MAP kinase isozymes and that this modification of these proteins appears to increase their enzymatic activity. Since MAP kinases are thought to be regulators of cellular activation, these kinases may be important targets for LPS action in macrophages. Together, these data provide additional support for the idea that protein tyrosine phosphorylation is an important signal transduction event in LPS-stimulated macrophages.

BZ 366 REGULATION OF EXTRACELLULAR SIGNAL REGULATED KINASES (ERKs) IN T CELLS

C.E. Whitehurst & T.D. Geppert, Harold C. Simmons Arthritis Research Center, Dept. of Int. Med., Univ. Texas Southwestern Med. Center, Dallas, TX 75235

ERK1 and 2 are serine/threonine kinases activated by threonine and tyrosine phosphorylation. They are activated by antibody-induced ligation of the TCR/CD3 complex and, therefore, are likely to play an important role in T cell activation. The current experiments examined the factors that regulate ERK activity in T cells. The malignant T cell line Jurkat were used as responding cells. The capacity of anti-CD3 to stimulate a recently described ERK activator was compared with its ability to stimulate ERK2. ERK2 activity was analyzed using an ERK2 specific immune complex kinase assay. The activity of the ERK activator was assessed in DEAE flow through fractions for the ability to activate recombinant ERK1 or 2 MBP kinase activity. Anti-CD3 stimulated both endogenous ERK2 and ERK activator activity. Both activities were detectable at 2 minutes and reached a maximum by 5 minutes. ERK2 tyrosine phosphorylation and activity decreased to baseline over 90 minutes, whereas the ERK activator activity remained relatively stable. The data suggest that inactivation of ERKs following anti-CD3 stimulation is mediated by the activation of tyrosine and possibly Thr/Ser phosphatases. To investigate the role of phosphatases in regulating ERK activity, we treated Jurkat cells with okadaic acid (OA), a specific inhibitor of Ser/Thr phosphatases (PP) 1 and 2A, or phenylarsine oxide (PAO), an inhibitor of tyrosine phosphatases, and examined ERK activator activity and ERK2 activity. OA and PAO each induced the phosphorylation and activity of ERK2, but had minimal effect on ERK activator activity, suggesting that they enhance ERK2 activity by directly inhibiting its threonine and tyrosine dephosphorylation. In support of this conclusion, a comparison of the phosphoaminoacid content of ERK1 and 2 from OA and anti-CD3 stimulated cells revealed that the ERKs from OA stimulated cells had a markedly higher ratio of phosphothreonine to phosphotyrosine. The data suggests, therefore, that the combined activities of an ERK activator and tyrosine and threonine specific phosphatases regulate ERK activity.

BZ 367 INHIBITION OF v-Mos KINASE ACTIVITY BY PROTEIN KINASE A. Y. Yang, B. Singh & R.B. Arlinghaus. Department of Molecular Pathology, University of Texas M.D.Anderson Cancer Center, Houston, Texas 77030

The v-mos gene of Moloney murine sarcoma virus (Mo-MuSV) encodes a serine/threonine protein kinase. Mutagenesis studies have shown that the protein kinase function of v-Mos correlates with its biological activity. The cellular mos gene product (c-Mos) is required for the initiation of meiosis I and II and as part of cytostatic factor is also responsible for cell cycle arrest of the egg in metaphase at the meiosis II. v-mos and c-mos gene products can substitute for each other in cellular transformation, induction of meiotic maturation and cell cycle arrest.

We have been studying the role of phosphorylation in the regulation of Mos kinase function. Previous studies from this laboratory have shown that v-Mos kinase activity is regulated positively by $p34^{cdc2}$ kinase and the protein kinase C pathway. Here we report that protein kinase A (PKA) upon activation can inhibit v-Mos kinase activity in transformed cells. To determine the effects of PKA, COS-1 cells expressing either $p37^{env-mos}$ (or its amino-terminal truncated form, structurally equivalent to $p39^{c-mos}$) or Mo-MuSV ts110 transformed NRK-6m2 cells expressing $P85^{gag-mos}$ were treated with forskolin, a known activator of adenyl cyclase. In all cases forskolin treatment inhibited Mos kinase activity in a dose-and time-dependent manner without affecting the steady state level of v-Mos. Purified PKA phosphorylated $p37^{env-mos}$ immunoprecipitated from insect cells and glutathione transferase-v-Mos fusion protein purified from Escherichia coli in vitro, suggesting that inhibition of v-Mos kinase by forskolin in vivo may be the direct consequence of its phosphorylation by PKA. There are eight PKA consensus phosphorylation sites in $p37^{env-mos}$, six of which are also present in mouse c-Mos. Based upon our results, we propose that the known inhibitory role of PKA in the initiation of oocyte maturation could be explained at least in part by its inhibition of Mos kinase.

BZ 368 MAP KINASE ACTIVATOR IN XENOPUS BELONGS TO A MULTI - MEMBERED FAMILY, Beverly M. Yashar[1], Clair Kelley[2], Karen Yee[2], Leonard I. Zon[2], Beverly Errede[1], [1]Department of Chemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, [2]Department of Hematology, Children's Hospital, Boston, MA, 02115

A variety of extracellular agents that influence cellular proliferation and differentiation results in the activation of MAP kinase. This activation occurs through a kinase cascade that ultimately leads to phosphorylation of MAP kinase. MAP kinase is part of an evolutionarily conserved family of proteins that includes ERK1 - 3, SPK1, FUS3 and KSS1 kinases. The latter two are members of a kinase cascade (STE20, STE11, STE7, FUS3, KSS1) involved in intracellular transmission of the signal that triggers the mating response in *S. cerevisiae*. Genetic studies have led to the prediction that STE7 is the activator of FUS3. Vertebrate MAP kinase activators which are related to STE7 have been recently purified. This similarity suggests that kinase cascades homologous to the one in *S. cerevisiae* are conserved in many, if not all, eukaryotes.

In an effort to clone the gene encoding the MAP kinase activator, we used PCR to identify cDNA clones expressed in *X. laevis* embryos related to the STE7 kinase family (STE7, BYR1, PBS2, WIS1). We recovered a group of distinct clones from both embryo and oocyte libraries. One of these unique cDNA clones (XMA1) encodes a predicted protein kinase with strong homology to STE7. Surprisingly, XMA1 is similar but distinct from the previously reported MAP kinase activators. XMA1 is highly expressed as a maternal mRNA, and temporally regulated during embryonic development. Whole embryo *in situ* analysis demonstrated high levels of expression in brain and muscle. Partial sequence of a second cDNA clone (XMA2) identified yet another potential MAP kinase activator. The strong amino acid sequence similarity of XMA1, XMA2 and MAP kinase activator suggests that these three are related members of a family of kinases involved in the activation of MAP kinase.

Ras

BZ 400 IDENTIFICATION OF A RAS-RELATED PROTEIN PHOSPHORYLATED BY c-AMP-DEPENDENT PROTEIN KINASE DURING CHEMICALLY-INDUCED DIFFERENTIATION OF MURINE ERYTHRO-LEUKEMIC CELLS. Gerry R. Boss, Jurgen S. Scheele, Lawrence A. Quilliam and Renate B. Pilz. Depts. of Medicine and Chemistry, Univ. of Calif., San Diego, La Jolla, Ca 92093-0652

Murine erythroleukemic (MEL) cells are a valuable model for studying hematopoietic cell differentition in vitro. We have recently found that MEL cells deficient in c-AMP-dependent protein kinase (A-Kinase) activity are severely impaired in their ability to differentiate (J.Biol.Chem. 267: 16161-16167, 1992). We now report on finding by two-dimensional (2-D) gel electrophoresis a low molecular weight A-Kinase substrate (pp22) that is phosphorylated during differentiation of parental cells that is not phosphorylated in the non-differentiating A-Kinase-deficient cells. We found that pp22 was recognized on immunoblots and was immunoprecipitated by the monoclonal antibody 142-24E05 which was raised against amino acids 96-118 of H-ras. PP22 was also recognized on immunoblots by the poly-clonal antibody R195 which was raised against rap 1a. We showed clearly, however, that pp22 does not co-migrate on 2-D gels with either purified H- or K-ras or rap 1a or 1b. Since only H- and K-ras and rap 1a and 1b are known to be A-Kinase substrates, it appears that pp22 is either a new ras-related protein or is a ras-related protein not previously known to be an A-Kinase substrate.

BZ 401 CHANGES IN TYROSINE PHOSPHORYLATION OF rasGAP-ASSOCIATED PROTEINS DURING MONOCYTIC DIFFERENTIATION OF CHRONIC MYELO-GENOUS LEUKEMIA CELLS. Jeng-Chung Cheng, P. Sivar Kumar, Bhanu Kannan, Jeffrey Clark and A. Raymond Frackelton, Jr, Department of Medicine, Brown University and Roger Williams Medical Center, Providence, RI 02908

Treatment of the blast-phase chronic myelogenous leukemia cell line, RWLeu4, with the phorbol esters, PMA, induces reversion to the more benign chronic-phase phenotype of rapidly and terminally maturing monocytes. This terminal differentiation results in i) markedly increased tyrosine phosphorylation of the 62-kDa protein that combines with proteins containing regions homologous to the SH2 domain of src; ii) increased tyrosine phosphorylation of the 120-kDa ras GTPase activating protein (GAP); iii) de novo appearance and tyrosine phosphorylation of a 100-kDa rasGAP; iv) changes in the association of p190 with rasGAP proteins; v) increased association of GAP with the 62-kDa protein. Increased tyrosine phosphorylation of p62 and the rasGAP was half-maximal 6 hrs after PMA exposure, well before the appearance of obvious differentiated characteristics such as adherence to plastic, ability to reduce NBT dye, and increases in CD45 and M01 antigens. Interestingly, PMA caused a striking increase in the fraction of ras in the presumedly active, GTP-bound state (from an already high 20% to 55%). Blocking isoprenylation of ras abrogated the ability of PMA to mature the CML cells.

BZ 402 Involvement of ras gene in insulin induced c-jun expression in human hepatoma cells. Chen-kung Chou[1,2] and Yea-Lih Lin[2], [1]Department of Medical Research, Veterans General Hospital. [2]Ins. of biochemistry, Yang-Ming Medical College, Taipei, Taiwan, R.O.C.
The potent fungal toxin lovastatin competitively inhibits the enzyme activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, and blocks the synthesis of mevalonate. Blocking mevalonate synthesis results in accumulation of nonisoprenylated p21ras protein and loss of transforming activity of p21ras proteins. It has been suggested that p21ras might be a connecting point for several growth factor mediated signaling pathways. In order to study whether p21ras can mediate signals induced by insulin to regulate gene expression. We pretreated human hepatoma Hep3B cell with lovastatin to inhibit the isoprenylation of ras protein and then evaluated the effect of insulin on c-jun gene expression in this cells. Our results show that the induction of c-jun expression by the physiological concentration of insulin (5×10^{-9} M) was totally blocked by pretreatment of 20uM lovastatin. Futhermore, the effect of lovastatin treatment on the phorbol ester, TPA, induced c-jun expression was also studied. We found that lovastatin treatment also block TPA induced c-jun gene expression in Hep3B cells. These results are consistent with our previous observation that insulin and TPA may share the same protein kinase C dependent pathway to induce c-jun gene expression in Hep3B cells. Our observations also suggest that p21ras may play an important role at the downstream of protein kinase C to mediate the effect of both insulin and TPA on the induction of c-jun gene expression in human hepatoma cells.

BZ 403 SELECTIVE SUPPRESSION OF ACTIVATED p21ras-DEPENDENT PROLIFERATION BY FLUOROMEVALONATE, Jennifer A. Cuthbert and Peter E. Lipsky, The University of Texas Southwestern Medical Center at Dallas, Dallas TX 75235

Stimulation of hematopoietic cells by the cytokine interleukin-3 (IL-3) activates normal p21ras whereas depletion of intracellular levels of p21ras suppresses proliferation induced by IL-3, suggesting that IL-3 signal transduction requires the function of p21ras. Post-translational modification of ras by prenylation is necessary for both membrane localization and transforming activity *in vitro*. However, the role of prenylation of p21ras in normal cell function has not been established. 6-fluoromevalonate (Fmev), an inhibitor of mevalonate metabolism, was used to block the prenylation of proteins in the interleukin-3 (IL-3) dependent cell line, FDC-P1 and FDC-P1 cells transformed with oncogenic ras (rasDC), which are IL-3 independent. Fmev inhibited prenylation of proteins as indicated by the concentration-dependent suppression of mevalonate incorporation. At 500 μM, Fmev suppressed [^{3}H] mevalonate incorporation into protein by 97.4 ± 0.5% (mean ± SEM, n=3). Fluorography of [^{3}H] mevalonate-labeled proteins, separated by SDS-polyacrylamide gel electrophoresis, was used to examine prenylation of specific proteins. [^{3}H] mevalonate labeled a number of proteins in both cell lines including the Mr ~21,000 ras protein. Fmev completely suppressed prenylation of this and all other proteins. Blocking prenylation of proteins with Fmev (500 μM) did not inhibit DNA synthesis in control FDC-P1 cells cultured with IL-3. In contrast to the effect noted with the parent cell line, suppression of prenylation mediated by Fmev (500 μM) significantly inhibited DNA synthesis by the IL-3 independent rasDC cells. Fmev-blocked rasDC cells however were able to proliferate in response to IL-3, indicating that the transformed phenotype rather than the proliferative machinery of the cells was altered by Fmev. These results indicate that prenylation of normal cellular ras is not required for IL-3 signal transduction or proliferation. In contrast, prenylation of oncogenic ras appears to be necessary for maintenance of the transformed phenotype of these cells.

BZ 404 CHARACTERIZATION OF A GUANINE NUCLEOTIDE DISSOCIATION STIMULATOR FOR A *Ras*-RELATED GTPASE. Barton W. Giddings, Charles F. Albright, Joana Liu and Robert A. Weinberg. Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142.

ras-related GTPases participate in signaling for a variety of intracellular processes. The cycling of these GTPases between the GTP-bound form and the GDP-bound form is partially controlled by guanine nucleotide dissociation stimulators (GDS). We report the molecular cloning of *ralGDS*, a new member of the *CDC25* family of GDS proteins. Using the cloned gene, we purified the *ralGDS* protein from insect cells which were infected with a recombinant baculovirus. We find that the purified *ralGDS* protein catalyzes the dissociation of guanine nucleotides from the *ralA* and *ralB* GTPases at a rate at least 30-fold faster than the rate without the *ralGDS* protein. *ralGDS* is at least 20-fold more active on the *ralA* and *ralB* GTPases than on any other GTPase tested, including other members of the *ras* family (*H-ras, N-ras, K-ras, R-ras, rap1a, rap2*), members of the *rho* family (*rhoA, rhoB, and CDC42-Hs*), and members of the *rab* family (*rab3a and ypt1*).

BZ 405 ACTIVATION OF RAS PROTEIN IN RESPONSE TO IL-6, Hiroshi Koide, Takaya Satoh, Masaaki Murakami[1], Tetsuya Taga[1], Tadamitsu Kishimoto[1], and Yoshito Kaziro, DNAX Research Institute, Palo Alto, CA94304 and [1]Institute for Molecular and Cellular Biology, Osaka University, Japan
Ras protein plays an important role in regulating cell growth in fibroblasts and differentiation in PC12 cells. It binds guanine nucleotides and cycles between an active GTP-bound form and an inactive GDP-bound form. We have shown that stimulation with IL-2, IL-3 and GM-CSF promotes the accumulation of an active GTP-bound form of Ras in hematopoietic cells. In the present study, we tested whether IL-6 can also activate Ras using two hematopoietic cell lines, KT3 and M1 cells. In both cells, accumulation of the GTP-bound form of Ras was induced in response to IL-6. The kinetics of Ras•GTP accumulation were much slower than in the case of IL-2 stimulation, the maximum being reached more than 10 min after the stimulation. These results suggest that Ras is involved in the signal transduction pathway of IL-6. We found that LIF, in addition to IL-6, can activate Ras in M1 cells, suggesting that gp130, which is the common component of the receptors for IL-6 and LIF, is responsible for Ras activation in the IL-6 system. IL-6 promotes the growth of KT3 cells while it induces the differentiation of M1 cells. To see whether Ras is essential for growth and/or differentiation in hematopoietic cells, we are presently examining the effect of a dominant inhibitory Ras mutant ([Asn^{17}]Ras) on these two types of cells.

BZ 406 PHOSPHORYLCHOLINE AND DIACYLGLYCEROL INDUCED BY SERUM AND *ras* ONCOGENES ARE NOT GENERATED BY A PHOSPHOLIPASE C. INVOLVEMENT OF PHOSPHOLIPASE D AND CHOLINE KINASE.

Juan Carlos Lacal, Amancio Carnero and Antonio Cuadrado. Instituto de Investigaciones Biomédicas, Madrid, Spain.

Mitogenic induction of NIH-3T3 fibroblasts with growth factors or *ras* oncogenes is associated with an increase in the levels of phosphorylcholine. This metabolite can be generated as a result of direct activation of either choline kinase or a phosphatidylcholine-specific phospholipase C (PC-PLC) or by more complex alternative pathways, such as activation of phospholipase D followed by choline kinase. Recent evidence has implicated a PC-PLC as the sole responsible for the generation of both phosphorylcholine and *dicylglycerol in cells* stimulated by growth factors and in *ras*-transformed fibroblasts. By using specific inhibitors, we have been able to disect the generation of phosphorylcholine from that of diacylglycerol in serum-treated and in *ras*-transformed NIH-3T3 cells. Our results indicate that neither serum or *ras* oncogenes generate phosphorylcholine and diacylglycerol in a PLC-dependent fashion. Instead, a PC-specific phospholipase D is primarily involved in the generation of both metabolites as a first step in a more complex signalling pathway which also involves choline kinase (CK)-dependent conversion of choline into phosphorylcholine. Thus, activation of PLC may not be related to regulation of cell proliferation in NIH-3T3 cells. Moreover, the presence of an active CK enzyme is a critical step in the mitogenic activity of growth factors, indicating that phosphorylcholine may be a novel second messenger molecule in regulation of cell proliferation. This notion is consistent withthe evidence that a number of transformed cells show an increase of phosphorylcholine.

BZ 407 BIOCHEMICAL AND IMMUNOLOGICAL CHARACTERIZATION OF THE CATALITIC DOMAIN OF A MOUSE RAS SPECIFIC GNRP .

E.Martegani, P.Coccetti, C. Ferrari, M.Vanoni, R.Zippel L.Alberghina, E.Sturani. Dipartimento di Fisiologia e Biochimica Generali, Universita degli Studi di Milano, 20133 Milano, Italy.

The percentage of Ras proteins in the active GTP bound state is determined by the balance between GTP hydrolysis and exchange of bound GDP. While the former process is mediated by GTPase Activating Proteins (GAPs) the latter process require the action of Guanine Nucleotide Releasing Proteins (GNRPs). Prototype of such function is represented by the product of the *S.cerevisiae CDC25* gene. We have previously cloned by functional complementation of a yeast *cdc25* mutant a partial sequence of a mouse CDC25 homolog .This fragment encodes the C-terminal domain of the mouse CDC25 (last 286 aa), shares a high homology with yeast Cdc25 and has a catalytic activity on ras proteins when tested as purified fusion protein in an in vitro sistem. In order to investigate the in vivo function of this catalytic domain we have carried out transient transfection assays on mammalian cells demonstrating that it is able to induce transactivation of a fos promoter in a ras dependent manner. Experiments are underway to define the minimal essential catalytic domain of the mouse GNRP able to complement yeast *cdc25* mutants and to transactivate the fos promoter in mammalian cells. Antibodies raised against the purified fusion protein allow to detect in mouse brain a protein of 130 kDa while two proteins of 75 and 95 kDa are recognized in NIH-3T3 fibroblasts. Further characterization of the different products both in different tissues and in cell lines has been undertaken.

BZ 408 REGULATION OF *ras* AND GAP ACTIVITY IN MAMMALIAN CELLS, Frank McCormick, Gideon Bollag, Paul Polakis, Simon Cook, Robin Clark and George Martin, Onyx Pharmaceuticals, 3031 Research Drive, Richmond, CA 94806.

Ras p21 activates a cascade of serine threonine kinases in response to various extracellular signals, including receptor tyrosine kinases and mitogenic G-proteins. *Ras* p21 is regulated by GDS proteins (guanine nucleotide dissociation stimulators) and GAPs (GTPase activating proteins). Two types of GAPs are known, p120-GAP and neurofibromin. The latter GAP is regulated by lipids, and through association with tubulin. p120-GAP associates with certain tyrosine kinase receptors, and is phosphorylated on tyrosine as a result. This protein also associates with the tyrosine phosphoproteins p190 (shown by Settleman and colleagues to encode a rhoGAP) and p62 (an RNA binding protein). Details of these interactions will be discussed. *Ras* p21 activity in cells may also be regulated by the related protein rap1A (also known as Krev1), which antagonizes *ras* transformation. We have investigated the mechanism by which rap1A blocks *ras* function, and shown that it inhibits *ras*-dependent activation of serine/threonine kinases without affecting signals upstream of *ras* p21. The possible physiological role of rap1A will be discussed.

BZ 409 EXPRESSION CLONING OF cDNAs FOR A NOVEL ONCOGENE, *ect2*, ENCODING A CANDIDATE ACTIVATOR OF SMALL GTP-BINDING PROTEINS

Toru Miki, Cheryl L. Smith,[1)] and Timothy P. Fleming[2)]
Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD 20892.
Present address: 1) University of California, Berkeley; 2) Washington University School of Medicine, St. Louis.

Using an expression cloning system with capability of efficient plasmid rescue from mammalian cells (Miki *et al.*, Gene *83*, 137-146, 1989; Miki *et al.*, PNAS *88*, 5167-5171, 1991), we constructed a keratinocyte cDNA library and introduced the DNA into fibroblasts. Several transformed foci were obtained following transfection. Characterization of the cDNA clones rescued from these transformants revealed that three distinct transforming cDNAs, *ect1*, *ect2*, and *ect3*, were isolated. The *ect1* cDNA was found to encode the keratinocyte growth factor receptor (Miki *et al.*, Science *251*, 72-75, 1991; Miki *et al.*, PNAS *89*, 246-250, 1992). Here, we report characterization of the *ect2* gene.

The *ect2* plasmid contained a 2.8-kb cDNA insert. This cDNA was shown to have been activated by amino-terminal truncation of the normal product, since the full-length *ect2* cDNA clones isolated by library screening did not show detectable transforming activity. The predicted ect2 protein sequence exhibited similarity within a central core of 255 amino acids with the products of the breakpoint cluster gene, *bcr*, the yeast cell cycle gene, *CDC24*, and the *dbl* oncogene. Each of these genes encodes regulatory molecules for rho-like small GTP-binding proteins. Thus, *ect2* represents a new member of an expanding family, whose products can exhibit transforming properties and interact with *rho*-like proteins of the *ras* superfamily.

BZ 410 SUPPRESSION OF THE BIOLOGICAL ACTIVITY OF ONCOGENIC BUT NOT NORMAL RAS BY MUTANT NEUROFIBROMATOSIS TYPE 1 (NF-1) GENES WITH SINGLE AMINO ACID SUBSTITUTIONS

Masaru Nagamine, Masato Nakafuku, Akihira Ohtoshi, Kazuma Tanaka*, and Yoshito Kaziro, DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304-1104, *Department of Biology, Faculty of Science, University of Tokyo, Tokyo 113, Japan

NF-1 was first identified as a gene which is responsible for the pathogenesis of a human genetic disorder, neurofibromatosis type 1. The cDNA cloning revealed that its putative protein product has a domain which shows a significant sequence homology with mammalian Ras GTPase activating protein (GAP) and two yeast *S.cerevisiae* proteins, Ira1 and Ira2. This GAP-related domain of the NF-1 gene product (NF1-GRD) has been shown to stimulate GTPase activity of normal human and yeast Ras proteins, but not of oncogenic mutant Ras. Thus, NF1-GRD complements *ira*-deficient yeast, and can suppress the heat shock-sensitive phenotype of *ira*$^-$ cells, but not the same phenotype of $RAS2^{Val19}$ and $RAS2^{Leu68}$ (correspond to human ras^{Val12} and ras^{Leu61}, respectively) cells. We screened a pool of mutagenized NF-1 expression plasmids, and obtained several mutant NF-1 cDNA clones which can suppress the phenotype of $RAS2^{Val19}$ cells. Among these clones, one clone (NF201) suppressed $RAS2^{Leu68}$ as well as $RAS2^{Val19}$, while the other clones (NF203, NF204) preferentially suppressed one of these two activated Ras alleles. Preliminary experiments have demonstrated that the expression of NF201 cDNA in mammalian cells inhibits the activation of c-*fos* promoter in response to c-Ha-ras^{Val12}. Sequence analysis has identified single amino acid substitution in each clone which is located in one of the conservative regions of NF1-GRD. Biological and biochemical properties of these mutant NF-1 will be discussed.

BZ 411 ACTIVATION OF RAS IN RESPONSE TO DIFFERENTIATION FACTORS IN RAT PHEOCHROMOCYTOMA PC12 CELLS,

Masato Nakafuku, Takaya Satoh, and Yoshito Kaziro, DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304-1104

Ras is involved in neuronal differentiation of rat pheochromocytoma PC12 cells. PC12 cells are adrenal chromaffin-like cells which undergo differentiation to sympathetic neuron-like cells in response to nerve growth factor (NGF), fibroblast growth factor (FGF) and interleukin(IL)-6. We found that these differentiation factors induce an accumulation of an active Ras·GTP complex. The percentage of Ras·GTP increased from 6% to 24% after 2 min stimulation with NGF, and the high level of Ras·GTP was maintained for at least 16 h. On the other hand, FGF and IL-6 induced about 3-fold increase of Ras·GTP, which returned to the basal level within 60 min. These observations provide direct evidence that activation of Ras is involved in signal transduction from these differentiation factors. In addition, it was found that epidermal growth factor (EGF), transforming growth factor-α (TGF-α), insulin, and insulin-like growth factor-I also activate Ras in PC12 cells under the same conditions. Although EGF was previously reported to stimulate proliferation rather than differentiation of PC12 cells, we could observe neuronal differentiation in response to EGF and TGF-α when the cells were cultured on a chemically coated plate. After 2 weeks exposure to these factors, the cells extended long and branched neurites, which was associated with the induction of neurofilament proteins. Thus, in all cases, factor-dependent activation of Ras leads to the induction of differentiation in PC12 cells. A tyrosine kinase-specific inhibitor, genistein, inhibited the increase of Ras·GTP induced by NGF and other factors. On the other hand, down-regulation of protein kinase C (PKC) by prolonged treatment with TPA, which sufficiently blocked TPA-induced Ras activation, did not abolish the formation of Ras·GTP by NGF. These results suggest that tyrosine kinases rather than PKC play a major role in the NGF-induced activation of Ras.

BZ 412 ACTIVATION OF RAS IN T CELLS AND POSSIBLE CROSS-TALKS BETWEEN G PROTEIN AND RAS PROTEIN SIGNALING SYSTEMS,

Tomokazu Ohtsuka, Takaya Satoh and Yoshito Kaziro, DNAX Research Institute, Palo Alto, CA 94304

Ras protein, a member of a family of signal-transducing GTP-binding proteins, plays an important role in growth and differentiation signaling processes. The activity of Ras is regulated by its bound nucleotides. When Jurkat cells are stimulated by PHA, an anti-CD3 antibody (OKT3) or PMA, the formation of the active GTP-bound Ras is stimulated. The level of Ras•GTP increases very rapidly to reach plateau within 5 minutes at 30 °C, and then starts to decrease to return to the initial level by 30 minutes. HerbimycinA, a specific inhibitor of tyrosine kinases inhibited the formation of Ras•GTP by PHA or OKT3 but not by PMA, suggesting that PHA and OKT3 utilize a certain non-receptor type tyrosine kinase to activate Ras. Cholera toxin catalyzes ADP-ribosylation of Gsα protein, which stimulates the activity of adenylate cyclase. When Jurkat cells were pretreated with cholera toxin, the stimulation of formation of Ras•GTP by PHA or OKT3 was inhibited, while that by PMA was not affected. This effect was not mimicked by dibutyryl cyclic AMP, suggesting that Gsα may modulate the Ras signaling system through the mechanism which does not involve the activation of adenylate cyclase.

BZ 413GENETIC INHIBITION OF p21^{RAS} FARNESYL TRANSFERASE.

George C. Prendergast, Joseph Davide, Ronald Diehl, Astrid Kral, Jackson Gibbs, Charles Omer, and Nancy Kohl. Department of Cancer Research, Merck Research Laboratories, West Point PA 19486.

Mutation of the Ras C-terminus prevents isoprenylation by farnesyl transferase and subsequent membrane insertion of the Ras protein, thereby eliminating its transformation activity. Based on these findings, it has been proposed that FTase represents a novel therapeutic target in tumors where Ras plays an etiologic role. However, the biological foundation for this proposal is uncertain. First, the consequences for animal cell physiology in cells where FTase activity is inhibited have not been examined. Second, though experiments performed in yeast indicate that Ras modification by FTase is necessary for Ras biological activity further evaluation of the biological phenotypes associated with specific inhibition of FTase in animal cells is required. Finally, if inhibition of FTase can be shown to suppress Ras function, it will be important for therapeutic reasons to determine whether the inhibition is cytotoxic or cytostatic for transformed cell growth.

To address these issues, therefore, we have sought to genetically inhibit FTase activity in normal cells and in cells transformed by Ras and other oncogenes. We have recently isolated cDNAs for the human FTase α and β subunits and are currently exploring antisense and dominant negative approaches to inhibit FTase activity *in vivo*.

BZ 414 Involvement of p21ras in transforming growth factor β1 (TGFβ1)-mediated growth inhibition.

Kumar B. Reddy, Steve Dobrowolski*, Dennis Stacey*, and Philip H. Howe. Depts. of Cell Biology and Molecular Biology*, Cleveland Clinic Research Institute, Cleveland, OH 44195.

TGFβ1 is a potent inhibitor of mink lung epithelial cell (CCL64) growth in culture. The observation that many transformed epithelial cells contain oncogenic forms of $p21^{ras}$ and have developed a resistance to TGFβ1 suggests that $p21^{ras}$ may mediate growth inhibition by TGFβ1. We transfected CCL64 cells with a mutated Ha-*ras* oncogene and investigated subsequent transfectants for their growth inhibitory response to TGFβ1. The cells display a transformed morphology, grow spontaneously under anchorage-independent conditions, and acquire a complete resistance to growth arrest by TGFβ1 despite displaying all TGFβ1 receptor types. Since we have previously demonstrated that modulation of $p34^{cdc2}$ kinase is a marker for TGFβ1 growth inhibition, we investigated $p34^{cdc2}$ activity in the CCL64 transfected clones. The results show that in the control CCL64 cells TGFβ1 regulation of $p34^{cdc2}$ activity is maintained. In the *ras* transfected cells $p34^{cdc2}$ phosphorylation and histone H1 kinase activity is significantly increased and regulation by TGFβ1 is lost. More direct support for the involvement of $p21^{ras}$ in TGFβ1 signaling is demonstrated by our microinjection studies in CCL64 cells. Injection of Ha-*ras* p21 into TGFβ1-arrested cells overcomes TGFβ1 growth inhibition and allows S phase entry. Cells released from TGFβ1 growth inhibition and microinjected with anti-$p21^{ras}$ antibody remain arrested in late G1 and do not enter S phase. In addition, TGFβ1 is shown to regulate the activation state of endogenous $p21^{ras}$ as measured by the proportion of GTP bound $p21^{ras}$. These results clearly identify $p21^{ras}$ as a downstream mediator of TGFβ1 signaling. Its activation state is negatively regulated by TGFβ1 and its overexpression leads to the uncoupling of TGFβ1-mediated growth arrest.

BZ 415 MUTAGENESIS OF A CONSERVED MOTIF IN RAS GAP

Richard H. Skinner, Nichola J.E. Johnson, Karen Chave, Geof Brownbridge#,Keith Moore#, Martin Webb# & Peter N. Lowe.
Dept. of Cell Biology, Wellcome Research Laboratories, Beckenham, Kent BR3 3BS, UK. and #National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

To date, four different proteins have been identified that stimulate the GTPase activity either of mammalian or yeast Ras proteins; GAP, IRA1, IRA2 and NF-1. Comparing the sequences of the catalytic domains of these proteins, one region (block 9 of ref. 1) appears to be particularly conserved and contains the most striking cluster of identical residues in the form of an invariant motif FLRXXXPA. We have previously reported the results of preliminary mutagenesis experiments on this motif, in which we identified residues L902 and R903 of Ras GAP as being critical for function[2]. Here we present a fuller characterisation of this motif and show, using a novel fluorescence anisotropy method for measuring binding of Ras to other proteins, that subtle structural perturbations in this region profoundly impair the catalytic efficiency of GAP without affecting its affinity for Ras, whereas more radical changes impair binding. The implications of these results for the molecular mechanism of GAP stimulation of Ras GTPase are discussed.

1. Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M. & Collins, F. (1990) Cell 63, 851-9.

2. Skinner, R.H., Bradley, S.,Brown, A.L., Johnson, N.J., Rhodes, S., Stammers, D.K. & Lowe, P.N. (1991) J. Biol. Chem. 266, 14163-6.

BZ 416 INHIBITION OF SUPEROXIDE PRODUCTION IN HUMAN B LYMPHOCYTES BY RAC ANTISENSE OLIGONUCLEOTIDES, **Aimé Vazquez, Olivier Dorseuil, Paul Lang, Jacques Bertoglio, Gérald Leca and Gérard Gacon, INSERM U.131, 32 rue des carnets 92140 Clamart, INSERM U.257, Paris and INSERM U.333, Villejuif, France.**

Rac 1 and Rac 2 gene products are small GTP binding proteins showing 92% homology to each other. According to recent studies performed in cell free systems, rac 1 and Rac 2 proteins may be involved in the activation of NADPH-oxydase, the superoxide-generating enzymatic complex active in phagocytes. Epstein-Barr Virus (EBV) transformed B lymphocytes which express rac 1 and rac 2 genes, also efficiently release superoxide anions when triggered by various cell surface stimuli. To investigate the regulatory role of rac proteins in living cells, we analysed superoxide production in response to cross-linking of surface immunoglobulins or phorbol ester treatment, in human EBV-transformed B lymphocytes pretreated with rac sense or antisense oligonucleotides. We show that i) the rac protein content estimated by immunoblotting can be decreased by 60% in rac antisense pretreated cells and ii) a strong (50-60%), dose dependent inhibition of superoxide production is observed in antisense pretreated cells whereas cells pretreated with sense oligonucleotide are unaffected. These data show that, in whole cells, superoxide production is modulated by the rac protein content, thus demonstrating the physiological role of rac proteins in the regulation of NADPH-oxidase.

Second Messengers

BZ 417 ONCOGENICITY OF AKR MCF MURINE LEUKEMIA VIRUS CORRELATES WITH INDUCTION OF CHRONIC PHOSPHATIDYLINOSITOL SIGNAL TRANSDUCTION, Ahmad M. Al-Salameh and Miles W. Cloyd, Department of Microbiology, University of Texas Medical Branch, Galveston, TX 77555-1019. Naturally-occurring recombinant murine leukemia viruses (MuLV), termed mink cell focusing-inducing (MCF) viruses, are the proximal leukemogens in spontaneous thymic lymphomas of AKR mice. The mechanism by which these viruses transform lymphocytes is not clear. Previous studies have implicated either integrational activation of proto-oncogenes, chronic autocrine immune stimulation, and/or autocrine stimulation of growth factor receptors (*e.g.*, IL-2r) via binding of the viral *env* glycoprotein (gp70) to these receptors. Any one of those events could also involve activation of second messenger signaling pathways in the cell. We examined whether infection with oncogenic AKR-247 MCF MuLV induced transmembrane signaling cascades in thymocytes of AKR mice. Cyclic AMP levels were not changed, but there was enhanced turnover of phosphatidylinositol phosphates, with concomitant increased in diacyglycerol and inositol 1,4,5-triphosphate. Thus, phospholipase C activity was increased. Protein kinase C activity was also elevated in comparison to uninfected thymocytes. These events occurred in parallel with MCF expression in the thymus, and were chronically maintained thereafter. No changes in phospholipid turnover occurred in an organ which did not replicate the MCF virus (spleen), nor in thymocytes of AKR mice infected with a thymotropic, non-oncogenic MCF virus (AKV-1-C36). Therefore, only the oncogenic MCF virus induced phosphatidylinositol signal transduction. Flow cytometric comparison of cell surface gp70 revealed that AKR-247 MCF infected thymocytes expressed more MCF gp70 than did thymocytes from AKV-1-C36 MCF infected mice, suggesting that certain threshold quantities of MCF *env* glycoproteins may be involved in this signaling. This type of signal transduction is not induced by stimulation of the IL-2 receptor, but is involved in certain oncogene systems (*e.g.*, *ras*, *fms*). Its chronic induction by oncogenic MCF MuLV may thus initiate thymocyte transformation.

BZ 418 SIGNALLING VIA THE CELL SURFACE GANGLIOSIDE GM1 IN HUMAN T CELLS : INDUCTION OF A CELL CALCIUM MOBILIZATION AND INFLUX BY A PATHWAY INDEPENDENT OF EARLY TYROSINE PHOSPHORYLATIONS AND OF INOSITOL PHOSPHATES PRODUCTION, Georges BISMUTH, Helene GOUY and Philippe Deterre, Laboratoire d'Immunologie Cellulaire et Tissulaire, CNRS URA 186, CERVI, Paris, France.

Signalling via cell-surface ganglioside GM1 in human Jurkat T lymphocytes leads to a rapid increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$). This is observed in wild-type Jurkat cells as well as in a Jurkat cell variant lacking GM1 after its reconstition with exogeneous gangliosides. The observed Ca^{2+} response is sustained in the presence of 1mM extracellular Ca^{2+}, while it becomes transient in Ca^{2+}-free medium suggesting both activation of the release of intracellular Ca^{2+} stores and influx from extracellular spaces. Stimulation via GM1 empties the CD3-Ins (1,4,5)P_3 sensitive intracellular Ca^{2+} pools. Thus it appears that the two pathways mobilize Ca^{2+} from common Ca^{2+} pools. However, no significant increase in total inositol phosphates or in Ins (1,4,5)P_3, measured by HPLC or radio receptor assay, can be detected after GM1 stimulation. Importantly, we do not find in western-blot studies any detectable early tyrosine phosphorylations induced via GM1. This apparently also concerns the γ-1 isoform of phospholipase C which tyrosine phosphorylation is not detectable in immunoprecipitation experiments. However, GM1 stimulation results in IL2 production comparable to that obtained after CD3 activation. Altogether, these data show that cell-surface gangliosides GM1 may act as a signalling molecule in human T lymphocytes, a finding of importance when considering a possible function for membrane carbohydrate structures in T cell-recognition systems.

BZ 419 **BASIC FIBROBLAST GROWTH FACTOR IS A NUCLEOTIDE BINDING PROTEIN**, Nathalie Boulle, Patrick Auguste, Risē Matsunami, and Andrew Baird, The Whittier Institute for Diabetes and Endocrinology, Department of Molecular and Cellular Growth Biology, 9894 Genesee Avenue, La Jolla, CA 92037

Basic fibroblast growth factor (bFGF) is characterized by its high affinity for heparin and numerous mitogenic and non-mitogenic effects on a wide variety of cell types in various tissues. Due to an almost ubiquitous distribution and its pluripotent biological effects, we are attempting to identify the mechanisms that regulate basic FGF activity *in vivo*. Our previous studies (*PNAS 86*:3174, 1989; *J. Cell Biol. 108*:374, 1990; *Molec. Endocrinol.* 5:1003, 1991) have concentrated on one potential mechanism that could regulate basic FGF: phosphorylation. Here, we report this growth factor can also bind nucleotides.

SDS-PAGE and autoradiography show radiolabelled ATP ([α^{32}- or γ^{32}-P]-ATP) binds to basic FGF in a dose- and time-dependent manner. The association and dissociation kinetics are slow (within hrs) at room temp (Kd 10 mM) with ~0.6 mole of ATP bound per mole of bFGF at equilibrium. The binding of ATP to basic FGF is temperature and pH-dependent, and is highly sensitive to increasing salt concentrations (NaCl, $MgCl_2$ and $CaCl_2$). Specificity studies show [γ^{32}-P]-GTP binds bFGF with the same efficiency as ATP, and competition experiments using various mono-, di- or triphosphate nucleotides suggest a selective binding of the di- and triphosphate moieties of the nucleotides. This ability to bind ATP is shared by acidic FGF, KGF, and FGF6, but not by other growth factors tested (TNF, GMCSF, TGFα, TGFβ, IGF_I, IGF_{II}, EGF). The properties of ATP-bound FGF (ATP-basic FGF) have also been examined. Although the binding of ATP to basic FGF is inhibited by high concentrations of heparin (10 μg/ml), both basic FGF and ATP-basic FGF bind equally well to immobilized heparin and have the same proliferative effect on ACE cells. The significance of nucleotide binding to bFGF is not yet known, but given the high concentrations of ATP in cells, it may represent a new post-translational modification regulating bFGF activity *in vivo*. Alternatively, as it has been shown for PD-ECGF growth factor, this post-translational change may reflect a cytoplasmic, non-growth factor-related enzymatic activity of the basic FGF.

BZ 420 REGULATION OF THE 45- AND 55-kDA FORMS OF YEAST PHOSPHATIDYLINOSITOL 4-KINASE BY NUCLEOTIDES. George M. Carman and Rosa J. Buxeda. Rutgers University, New Brunswick, NJ 08903.

Phosphatidylinositol (PI) 4-kinase catalyzes the phosphorylation of PI to phosphatidylinositol 4-phosphate. This is the first step in the phosphatidylinositol signaling pathway, which has been shown to be important for cell growth and proliferation. Previous pulse-labeling studies suggested that the phosphorylation of PI was regulated by the energy charge of cells. We examined this regulation directly by using pure preparations of the 45- and 55-kDa forms of PI 4-kinase. Adenosine derivatives and nucleotides inhibited both forms of the enzyme with IC_{50} values ranging from 0.8 to 10 mM. The most potent inhibitor of the enzymes was MgADP. Detailed kinetic analyses of the enzymes showed that MgADP was a competitive inhibitor with respect to MgATP and a noncompetitive inhibitor with respect to PI. The *Ki* values for MgADP for the 45- and 55-kDa PI 4-kinases of 0.14 mM and 0.25 mM, respectively. These values were about 2-fold lower than the *Km* values for MgATP (0.36-0.5 mM). On the other hand, these *Ki* values were slightly higher than the cellular pool of ADP (0.1 mM). Thus, increases in cellular ADP levels and concomitant decreases in the energy charge could down-regulate PI 4-kinase activities and PI phosphorylation *in vivo*. Both forms of PI 4-kinase were also examined for their regulation by cytidine, thymidine, uridine, and guanosine nucleotides. Whereas the mono-, di-, and tri-phosphorylated derivatives of these nucleotides inhibited 55-kDa PI 4-kinase, nucleotides had no effect on 45-kDa PI 4-kinase. The most potent nucleotide inhibitor was CTP. CTP was a competitive inhibitor of 55-kDa PI 4-kinase with respect to MgATP with a *Ki* value of 1.5 mM. This *Ki* value was 4-fold higher than the *Km* for MgATP and 18-fold higher than the cellular pool of CTP. Thus, under normal growth conditions, CTP would not regulate 55-kDa PI 4-kinase activity. However, when CTP levels increase (e.g. inositol starvation), 55-kDa PI 4-kinase activity could be regulated by CTP *in vivo*. Regulation of 55-kDa PI 4-kinase activity by CTP may be related to overall phospholipid synthesis in yeast.

BZ 421 **EFECTS OF PHOSPHOLIPASE C ON GLUCOSE TRANSPORT AND PHOSPHORYLATION OF ENDOGENOUS PROTEINS IN RAT EPITROCHLEARIS MUSCLES *IN VITRO***, David T. Chu, Denise Barilla, Celia G. Aranda, and Douglas A. Young, Dept. of Diabetes, Sandoz Research Inst. East Hanover, NJ 07936

Skeletal muscle is responsible for the majority of glucose disposal *in vivo*. Insulin (INS) and contractile activity have been shown to stimulate muscle glucose transport and to promote translocation of GLUT4 glucose transporters (GT) to the plasma membrane. However, little is known about the mechanism of stimulation of glucose transport by muscle contraction. In this study, phospholipase C (PLC), which increases glucose transport in muscle in a manner similar to contraction, was used to evaluate the association between stimulation of glucose transport and phosphorylation of GLUT4 GT and other endogenous proteins in isolated epitrochlearis muscles. Muscle contraction, stimulated by electric current, induced a 2.5-fold increase in 3-O-methylglucose transport. INS (10 mU/ml) and PLC (0.6 U/ml) stimulated transport of 3-O-methylglucose 2.25- and 1.8-fold respectively. ^{32}P-prelabeled epitrochlearis muscles were treated with INS and PLC for 30 min. INS and PLC treatments reduced phosphorylation of the immunoprecipitated GLUT4 GT 12% and 30% respectively as compared to basal. INS treatment also reduced the phosphorylation of two endogenous proteins (18 and 16kDa). In contrast, PLC stimulated the phosphorylation of three endogenous proteins (30, 18.5 and 16kDa) and reduced the phosphorylation of a 15.5kDa protein. In conclusion: (1) INS, PLC and contraction increase 3-O-methylglucose transport, (2) INS and PLC reduce GLUT4 GT phosphorylation, and (3) INS and PLC induced different phosphorylation patterns in a number of endogenous proteins.

BZ 422 INS(1,3,4,5)P_4/INS(1,3,4,5,6)P_5 3-PHOSPHATASE: A TARGET FOR REGULATION BY PROTEIN PHOSPHORYLATION?

Andrew Craxton, Katsumi Nogimori and Stephen B. Shears, Inositol Lipids Section, Laboratory of Cellular and Molecular Pharmacology, NIEHS/NIH, Research Triangle Park, North Carolina 27709

There has been intensive research into the function and metabolism of Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 in relation to the control of Ca^{2+}-dependent protein phosphorylation. From this work it has emerged that an enigmatic Ins(1,3,4,5)P_4 3-phosphatase activity is widely distributed in a variety of cell-free systems. The conversion of Ins(1,3,4,5)P_4 to Ins(1,4,5)P_3 has the potential to play an important role in signal transduction, and interest in the 3-phosphatase was recently underscored by the observation that, in human platelets, treatment with phorbol esters activated the enzyme (Oberdisse *et al.*, *J. Biol. Chem.* 265:726-730, 1990).

We have purified the enzyme from rat liver to homogeneity and found that Ins(1,3,4,5,6)P_5 and InsP_6 are potent endogenous inhibitors of Ins(1,3,4,5)P_4 3-phosphatase activity by virtue of being competing high affinity substrates. This observation, and the high intracellular levels of Ins(1,3,4,5,6)P_5 and InsP_6, make it likely that these are the physiological substrates, such that the enzyme is unlikely to attack Ins(1,3,4,5)P_4 *in vivo*. Moreover, following reconstitution of the purified hepatic enzyme with protein kinase C, we were unable to show significant changes in the activity of this enzyme. We are currently using polyclonal antibodies to investigate the possibility of cell-specific isoforms of this enzyme, but in any case our data indicate that Ins(1,3,4,5)P_4 3-phosphatase is unlikely to be a widespread enzyme activity *in vivo*, and appears unlikely to be regulated by protein kinase C.

BZ 423 PHOSPHORYLCHOLINE AND DIACYLGLYCEROL INDUCED BY SERUM AND RAS ONCOGENES INVOLVES ACTIVATION OF PHOSPHOLIPASE D. Antonio Cuadrado, Amancio Carnero, and Juan Carlos Lacal. Instituto de Investigaciones Biomédicas, CSIC, Arturo Duperier 4, 28029 Madrid, Spain.

Mitogenic induction of NIH 3T3 fibroblasts with growth factors or ras oncogenes is associated with an increase in the levels of phosphorylcholine (PCho) and diacylglycerol (DAG). These metabolites may by generated directly by hydrolysis of phosphatidylcholine (PC) by a C type phospholipase (PC-PLC) or by other mechanisms such as activation of phospholipase D to generate choline and phosphatidic acid (PA) and subsequent activation of choline kinase and PA phosphatase. Recent reports have implicated a PC-PLC as the sole responsible for the generation of both PCho and DAG in cells stimulated with growth factors and in ras-transformed fibroblasts. By using specific inhibitors, we have been able to uncouple the generation of phosphorylcholine from that of diacylglycerol in serum-treated and ras transformed NIH 3T3 cells. Our results indicate that neither serum nor ras oncogenes generate phosphorylcholine and diacylglycerol in a PC-PLC dependent fashion. Instead, a PC-specific phospholipase D is primarily involved in the generation of both metabolites as a first step of a more complex signalling pathway.

BZ 424 Phosphatidylinositol 3-Kinase Associates with the Activated Erythropoietin Receptor. Damen, J., Mui, A.L.F., Pawson, T. & Krystal, G. The Terry Fox Laboratory, BC Cancer Research Centre, Vancouver, BC, V5Z 1L3, Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, M5G 1X5

The murine erythropoietin receptor (mEpR) is a member of the hemopoietin receptor superfamily which is characterized by the presence of 4 conserved cysteines and a Trp-Ser-X-Trp-Ser motif in its extracellular domain and the absence of a tyrosine kinase consensus sequence in its proline rich intracellular region. However, although it lacks tyrosine kinase activity, the mEpR, along with a number of other intracellular proteins, becomes transiently phosphorylated on tyrosine resides within minutes of binding Ep. We now show that one of these transiently phosphorylated proteins is the 85 kDa regulatory subunit of phosphatidylinositol 3-kinase (PI 3-kinase), a protein which has been shown to play an essential role in PDGF stimulated mitogenesis. Specifically, we have found, using anti-phosphotyrosine (αPY) immunoprecipitation and αPY immunoblotting techniques, that an 85 kDa protein is maximally phosphorylated on tyrosine residues within 10 min of incubation with Ep in Ba/F3 cells expressing the mEpR. Reblotting with antibodies to the 85 kDa subunit of PI 3-kinase revealed that this protein was p85α. Moreover, using biotinylated Ep (B-Ep) and streptavidin aqarose beads, followed by αp85α immunoblots, we found that the 85 kDa subunit of PI 3-kinase was specifically associated with the activated mEpR. In vitro PI 3-kinase assays of anti-mEpR immunoprecipitates and B-Ep associated preparations also demonstrated that PI 3-kinase activity was physically associated with the activated mEpR.

To determine whether this association was mediated through either of the two SH2 domains of the p85α, both SH2 domains as well as the SH2 domains of GTPase-activating protein (GAP) and phospholipase C-γl (PLC-γl) were expressed as GST fusion proteins in bacteria and tested for their ability to specifically bind the activated EpR. Our results clearly show that both SH2 domains of the 85 kDa subunit of PI 3-kinase, but not those of GAP or PLC-γl, bind tightly to the tyrosine phosphorylated EpR.

BZ 425 Effects of the Immune Suppressants FK-506, Cyclosporin A and Rapamycin on PHA Signalling in Human T Lymphocytes, David P. Dean, Joseph M. Merenda, Joseph P. Gardner, Jeffrey H. Hanke and Patricia A. Connelly, Department of Molecular Genetics, Pfizer Central Research, Groton, CT

FK-506 and Cyclosporin A form complexes with their respective immunophilins that have been shown to inhibit the calcium/calmodulin dependent phosphatase, calcineurin. It has been proposed that these complexes block a calcium-dependent signalling pathway essential for early gene activation in T lymphocytes. A third immune suppressant, rapamycin, binds to the FK-506 binding protein, does not inhibit calcineurin, but does inhibit a ribosomal S6 protein kinase. The present study used two dimensional gel electrophoresis to compare the effects of these three compounds on PHA-stimulated protein synthesis in human peripheral blood T cells. The expression of 307 proteins was quantitated and found to be induced relative to control in the presence of PHA. When inhibition of specific proteins was examined individually it was determined that co-administration of FK-506 and PHA inhibited by more than 50% the synthesis of 209 of the 307 proteins induced by PHA alone. In contrast, co-treatment with rapamycin and PHA inhibited the synthesis of only 11/307 proteins, 10 of which were also inhibited by FK-506 treatment. Seven of the 10 proteins repressed by both FK-506 and rapamycin are present at significant levels in untreated lymphocytes and thus induced only 4 - 8 fold by PHA. The remaining three proteins are highly induced by PHA (16 - 32 fold). Cyclosporin A was similar to but less efficacious than FK-506. The dramatic differences seen in protein synthesis profiles in PHA-treated human peripheral blood T cells following co-administration of FK-506 and cyclosporin A or rapamycin supports recently published data suggesting that FK-506 and rapamycin inhibit T cell proliferation at different steps in the signal transduction pathway.

BZ 426 *IN VITRO* EFFECTS OF A NOVEL INSULIN-MIMETIC ON GLUCOSE TRANSPORT AND METABOLISM, LIPOLYSIS, AND PROTEIN PHOSPHORYLATION, Joseph L. Evans, Christian M. Honer, Barbara E. Womelsdorf, and Philip A. Bell, Diabetes Department, Sandoz Research Institute, East Hanover, NJ 07936

In this study, we examined the *in vitro* effects of a novel insulin-mimetic (NIM), a microbial metabolite originally identified by its ability to acutely stimulate glucose transport in the absence of insulin, on a number of insulin-regulated processes. In isolated rat adipocytes, 3T3-L1 adipocytes, and L6 myocytes, NIM (2.5-10 μM) stimulated glucose transport by approximately 3-6-fold, with a maximally effective concentration of 5 μM. NIM inhibited adenosine deaminase-stimulated lipolysis (IC_{50} = 2.4 μM) in rat adipocytes, and was antilipolytic under other experimental conditions where insulin is uniquely active. In isolated rat hepatocytes, NIM inhibited endogenous glucose production dose-dependently (IC_{50} = 8 μM), and stimulated glycogen synthesis by approximately 25%. In ^{32}P-labeled 3T3-L1 adipocytes, both insulin and NIM acutely stimulated the phosphorylation of a number of cellular proteins, but only insulin produced an increase in the tyrosine phosphorylation of the ß–subunit of the insulin receptor and the insulin receptor substrate (IRS-1). In Fao hepatoma cells, insulin and NIM caused the rapid accumulation of a distinct set of phosphotyrosine-containing proteins, suggesting that NIM like insulin mediates at least some of its effects through the alteration of kinase and/or phosphatase activity. Consistent with this hypothesis, both insulin and NIM acutely stimulated protein phosphatase activity in rat adipocytes. These results suggest that NIM is interacting with a component of the insulin signalling pathway and is a unique tool with which to study the mechanism of insulin action. Identification of its molecular site of action might reveal a pharmacological target which overcomes or bypasses the post-binding defects which contribute to insulin resistance.

BZ 427 HORMONAL REGULATION OF CYTOSOLIC PHOSPHOLIPASE A_2 IN KERATINOCYTES, Gerhard Fürstenberger, Raimund Kast and Friedrich Marks, Department of Tissue Specific Regulation, German Cancer Research Center, W-69 Heidelberg, GERMANY

Eicosanoids have been shown to be critically involved in inflammatory processes and epidermal hyperproliferation in vivo induced by various physical and chemical stimuli. Moreover, a dysregulation of epidermal eicosanoid biosynthesis has been observed in benign and malignant hyperproliferative skin diseases. The rate-limiting precursor of eicosanoid biosynthesis is arachidonic acid which is released from the sn-2 position of phospholipids primarily by phospholipase A_2 (PLA_2).
Here we show that in keratinocytes the enzyme responsible for the selective release of arachidonic acid is a cytosolic PLA_2 ($cPLA_2$; 85 kd) which is stimulated by the proinflammatory mediator bradykinin (Bk) along a B_2-receptor-G-protein-dependent pathway and by the endogenous epidermal mitogen Transforming Growth Factor-α (TGF-α) through a EGF receptor tyrosine kinase-dependent pathway. The activation of $cPLA_2$ includes the translocation of the enzyme to the membrane and the stimulation of its catalytic activity. In Bk-stimulated cells the trans-location step was found to occur in the presence of increased cytoplasmic Ca^{2+} concentrations. Moreover, $cPLA_2$ activity in membrane fractions of Bk-stimulated cells was stimulated in the presence of non-hydrolysable GTP analogs indicating a G-protein-dependent step to be involved in the stimulation of the catalytic activity of $cPLA_2$. In contrast to Bk, basal cytoplasmic levels of Ca^{2+} appear to be sufficient for both TGF-α-induced translocation of $cPLA_2$ to the membrane and increase of the activity of the cytosolic enzyme. Stimulation of $cPLA_2$ activity by TGF-α but not by Bk stimulated the phosphorylation of the enzyme on tyrosine. The data suggest that EGF receptor-catalyzed tyrosine phosphorylation plays a crucial role in both translocation and activation of the enzyme.

BZ 428 AN INOSITOL MONOPHOSPHATASE GENE FROM TOMATO: REGULATION BY LITHIUM AND LIGHT, Glenda Gillaspy and Wilhelm Gruissem, Departtment of Plant Biology, University of California, Berkeley, CA 94720.
Breakdown of phospholipids in cell membranes can be controlled by external growth signals and results in production of the second messengers diacylglycerol (DAG) and inositol triphosphate (IP_3). This signalling cascade operates in animal cells in response to many stimuli, but it is not known how this cascade operates in plants, i.e. classical receptors and second messengers have not been found in plants. We have evidence that signalling does occur via the breakdown of phospholipids in plants from the cloning of a gene homologous to bovine inositol monophosphatase.This gene, tomato inositol monophosphatase (TIM), may function as its animal counterpart in breakdown of IP_3, thus recycling the intermediates neccesary for some signalling pathways. The clone was obtained from a young tomato fruit expression library and the gene encodes a 269 amino acid protein Southern analysis suggests there is at least one other DNA sequence similar to TIM in tomato. The entire cDNA when used as a probe in northern blots, detects an abundant level of TIM mRNA in young fruit which declines in ripening fruit. In seedlings TIM mRNA levels may be regulated by light.
To understand the function of the TIM gene,we are investigating the ability of an extract containing TIM recombinant protein to remove a phosphate from ^{14}C-labelled inositol monophosphate, and have found that the encoded enzyme is active. Preliminary results indicate that the recombinant enzyme is inhibited by high concentrations of lithium, and is activated by calcium. Purified recombinant protein is also being characterized.

BZ 429 PURIFICATION AND CHARACTERISATION OF MAMMALIAN INOSITOL LIPID - SPECIFIC PHOSPHOLIPASE C ISOZYMES EXPRESSED IN BACTERIAL CELLS, Rebecca S. Ginger, Amanda J. Carozzi, Peter J. Parker, Imperial Cancer Research Fund, 44, Lincolns Inn Fields, London, WC2A 3PX
Phosphatidylinositol lipid - specific phospholipase C (PtdIns-PLC) forms part of a signal transduction cascade in which agonist activated receptors are coupled to the activation of PtdIns-PLC. This enzyme catalyses the hydrolysis of phosphatidylinositol 4,5-bisphosphate to the second messengers inositol 1,4,5-trisphosphate (which mobilises intracellular calcium) and diacylglycerol (which binds and activates protein kinase C). Several isozymes have been cloned and purified. These isozymes have been grouped into three classes: β, γ and δ.
PtdIns-PLC δ1 has been expressed in bacteria as a fusion protein containing a short 22 amino acid lac z derived amino terminus. The soluble protein was then purified to homogeneity in a simple 3-step protocol and characterised to enable comparison with the eukaryotic enzymes. The catalytic properties of this enzyme closely resemble the range of characteristics reported for the mammalian enzyme purified from a variety of tissues and species.
Having successfully expressed one of the smaller isozymes of the PtdIns-PLC family, attempts have been made to generate catalytically active PtdIns-PLC β1 in a similar manner. Using a different expression vector system, purification and characterisation of this larger polypeptide have been possible. Studies examining various aspects of the regulation of this enzyme will be presented.

BZ 430 ROLE OF PROTEIN- AND PHOSPHOLIPID-KINASES IN CELL GROWTH AND TRANSFORMATION INDUCED BY G PROTEINS AND THEIR COUPLED RECEPTORS.

J. Silvio Gutkind, Gilda Kalinec and Ningzhi Xu. Molecular Signalling Group, Laboratory of Cellular Development and Oncology, NIDR, NIH, Bethesda, MD 20892.

We have used the family of human muscarinic acetylcholine receptors (mAChRs) expressed in NIH 3T3 cells as a model for studying the role of G protein-coupled receptors in cell growth and transformation. In this system, genes for mAChR subtypes (m1, m3, m5) which are coupled to the activation of phosphatidylinositol (PI) hydrolysis are transforming, whereas those (m2,m4) which are coupled to the inhibition of the adenylyl cyclase (AC) are not. Expression of a GTPase deficient α subunit of G_{i2}, which inhibits AC, fails to transform NIH 3T3 cells whereas expression of an activated mutant of G_q, which activates PI-hydrolysis, is weakly focus-forming in the same cells. Receptor-chimeras between transforming and non-transforming mAChRs which couple to PI-hydrolysis but fail to activate other signal transduction pathways are impaired as oncogenes. Thus, induction of G_q-PI hydrolysis appears not to be sufficient to account for the high transforming efficiency of certain G protein coupled receptors. These and other findings raise the possibility that cellular transformation might involve novel G protein-linked pathways.

Transforming G protein-coupled receptors can transduce potent mitogenic signals which are independent of conventional protein kinase C (PKC) but involve the c-raf serine-threonine kinase and probably the novel PKC-ζ isozyme. Activation of transforming mAChRs induces rapid tyrosine phosphorylation of cellular proteins including $p125^{FAK}$ and the p130 v-src substrate, involves the activation of PI-3 kinase, and affects $p21^{ras}$ and ras-GAP. Work in progress will be discussed.

BZ 431 KINETIC ANALYSIS OF PHOSPHATIDYLINOSITOL 3'-KINASE

David C. Heimbrook, Stanley Barnett, Lynette Miles, Steven M. Stirdivant, Janet Ahern, and Allen Oliff, Department of Cancer Research, Merck Research Laboratories, West Point, PA 19486

Phosphatidylinositol 3'-kinase (PI3'K) phosphorylates phosphatidylinositols (PI, PI-4P, and PI-4,5P_2) specifically at the 3'position of the inositol ring. Activation of PI3'K activity appears to be regulated by binding to activated tyrosine kinase growth factor receptors. This signalling pathway appears to be necessary for mitogenic activation mediated by a number of growth factor receptors. In order to identify specific inhibitors of this enzyme which might prove therapeutically useful, we initiated a detailed kinetic analysis of PI3'K purified from bovine brain. Initial studies suggested that the activity of some lipophilic inhibitors might be due to alteration of the affinity of PI3'K for the membrane interface, as has been previously proposed for phospholipase A_2 (F. Ramirez and M.K. Jain, "Proteins: Struct., Func., and Gen." 9 :229 (1991)). We conducted a detailed analysis of the kinetics of alternative substrate utilization and inhibitor activity for this enzyme, and observed that variations in the enzyme / membrane interface have a dramatic effect on PI3'K activity. These results suggest that inhibition of PI-3'K by lipophilic substrate analogs must be analyzed in the context of a biphasic catalytic system.

BZ 432 INVOLVEMENT OF PCI REGION IN TYROSINE KINASE-MEDIATED PLC-GAMMA ACTIVATION,

Yoshimi Homma, Department of Biosignal Research, Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho, Itabashi-ku, Tokyo 173, Japan

Recent studies have shown that recombinant Z (SH2/SH3) proteins derived from two isoforms of PLC-γ suppress the enzyme activity of four different PLC isoforms in a dose-dependent manner. This suppressive effect was very potent and stoichiometric. The kinetics studies showed that the suppression was non-competitive type. This suppression was not affected by heat treatment at 95 °C for 30 min, indicating that the primary structure would be important for the action of Z proteins. Comparative studies suggested that Z proteins, but not Src and phosphatidylinositol 3-kinase, possess adjacent to their SH2 and SH3 motifs, phospholipase C inhibitor (PCI) region that is involved in the suppression of phosphoinositide-hydrolyzing activity. A series of synthetic peptides identical with the sequence of proposed PCI region, including an octamer, YRKMRLRY, inhibited phosphoinositide hydrolysis induced by four different phospholipase C isoforms. On the other hand, suppressive effect of Z proteins was significantly diminished by their tyrosine phosphorylation induced by epidermal growth factor receptor. This change was not observed in a mutated Z protein whose tyrosine residue (771 or 783) was substituted by phenylalanine. These results demonstrate that PLC-γ contains the PCI sequence which is responsible for regulation of PLC-γ by tyrosine kinases, indicating that phospholipase C-γ is a self-regulating enzyme.

BZ 433 CALCIUM SENSITIVITY OF THE INHIBITORY ACTION OF LITHIUM ON INOSITOL POLYPHOSPHATE METABOLISM IN RAT CEREBRAL CORTEX

Stephen Jenkinson, Stefan R. Nahorski & R.A. John Challiss, Pharmacology Department, Medical Sciences Building, University of Leicester, Leicester, U.K.

A therapeutically relevant action of the monovalent cation lithium in CNS is its demonstrated ability to cause time-dependent inhibitions of agonist-stimulated inositol polyphosphate accumulations in cerebral preparations. In this study we have examined the calcium dependency of the inhibitory actions of lithium on Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 accumulations and the products of their metabolism in cerebral cortex slices.

Cross-chopped rat cerebral cortex slices were washed and incubated for 60 min with [^{3}H]inositol in Krebs-Hensleit buffer (KHB). After this period, slices were either transferred to fresh KHB or extensively washed in Ca^{2+}-free KHB. Slices were challenged with carbachol (CCH; 1 mM) + 1 mM LiCl for 20 min. Incubations were terminated and [^{3}H]inositol phosphate isomers resolved by h.p.l.c.

In the presence of LiCl, total [^{3}H]InsP_3 accumulation was reduced in low Ca^{2+} KHB (1-5 μM) compared to normal Ca^{2+} (1.3 mM) by about 30%, whereas no significant difference was observed in the absence of LiCl. Ins(1,4,5)P_3 accumulation was reduced from 3628 + 39 to 1638 + 238 d.p.m./50 μl of slices in the low Ca^{2+} medium, whereas Ins(1,3,4,5)P_4 accumulation was unaffected by $[Ca^{2+}]_o$. The inhibitory effects of LiCl on inositol polyphosphate accumulation were evident irrespective of $[Ca^{2+}]_o$; thus 1 mM LiCl resulted in 66% and 81% inhibitions of Ins(1,3,4,5)P_4 accumulation for incubations conducted in KHB containing 1.3 mM versus 5 μM calcium, respectively.

We conclude that although low extracellular Ca^{2+} may influence inositol phosphate generation and metabolism, the disruption of the phosphoinositide cycle by lithium is maintained under these conditions.

BZ 434 HIV-1 gp120 AND ANTI-CD4 INDUCED CROSSLINKING OF CD4-P56^{LCK} MODULATE ASSOCIATED PHOSPHATIDYLINOSITOL 3-KINASE AND PHOSPHATIDYL INOSITOL 4-KINASE ACTIVITIES, Rosana Kapeller, K. V. S. Prasad, Heinrich Repke, Jonathan S. Duke-Cohan, Lewis C. Cantley and Christopher E. Rudd, Dept. of Physiology, Tufts University Medical School, Boston, MA, 0211, Dept. of Pathology, Harvard Medical School and Divisions of Tumor Immunology and Retrovirology, Dana Farber Cancer Institute, Boston, MA, 02115

CD4 is a T cell surface antigen that binds to MHC class II molecules and is the receptor for the Human Immunodeficiency Virus 1 (HIV-1) glycoprotein gp120. CD4 is coupled to the protein-tyrosine kinase p56^{lck}, an interaction that is important for T cell activation. To date little is known about the signaling components downstream from the CD4-p56^{lck} complex . In this work, we show that CD4:p56^{lck} complex associates with significant levels of a phosphatidylinositol kinase (PI kinase). Furthermore, HIV-1 gp120 and anti-CD4 mediated CD4 cross linking induced a several fold increase in levels of anti-CD4 precipitable PI kinase activity. HPLC analysis demonstrated that both PI-3-P and PI-4-P were formed in the lipid kinase reaction performed on the immunecomplexes, indicating an increase in associated PI 3- and PI 4-kinase activities. We were also able to detect, on the anti-CD4 immunoprecipitates, the 85kDa subunit of PI 3-kinase by anti-p85 immunoblotting. CD4-p56^{lck} associated PI 4-kinase was five fold greater than PI 3-kinase activity. The ability of gp120 binding to modulate the association and/or activity of CD4-p56^{lck} linked PI kinases suggest that these activities may be involved in HIV-1 induced immune defects.

BZ 435 **Glucocorticoid-induced Diacylglycerol Kinase Homologue.** Theresa Klauck, Steve Harris and Susan Jaken, W. Alton Jones Cell Science Center, Lake Placid, NY 12946.

We have isolated a partial cDNA clone with homology to diacylglycerol kinase (DGK) from a glucocorticoid-induced hamster smooth muscle tumor cell (DDT1-MF2) library. The protein (p140) was originally identified in these cells as a 140 kDa phosphoprotein that cross-reacted with anti-α-PKC antibodies and was induced by glucocorticoids (triamcinolone acetonide, TAA). A p140 cDNA clone (2.6 kb) was isolated by immunoscreening a lambda expression library made from TAA-treated DDT1 cells with the α-PKC antibodies. Sequence analysis demonstrated an open reading reading frame of 1.3 kb which correlated with the size of the bacterially expressed protein (48 kDa). Neither the cDNA nor the deduced amino acid sequences had strong homology to PKC or to any other sequences in GenBank. The cDNA clone hybridized to a DDT1 cell TAA-induced message at 6.6 kb with weaker messages at 4.4 kb and >9.5 kb. Antisera were raised to a C-terminal peptide from the translated sequence and to the bacterially expressed fusion protein. Both recognized a 140 kDa TAA-induced protein in DDT1 cells, indicating that we have isolated a partial p140 cDNA clone. The library was rescreened with the cDNA clone and a larger, overlapping clone (4.0 kb) was isolated. Sequence analysis showed that the 5'end of the 4.0 kb clone has ~50% nucleotide and amino acid homology to a 3' region of porcine and human DGK which contains part of the putative catalytic domain . This region (porcine amino acids 306-509) is also conserved in a Drosophila DGK protein. Alignment of the human, pig, Drosophila and hamster proteins showed that, in general, the Drosophila and hamster sequences diverged from the human and porcine sequences at the same sites. It is possible that the consensus sequence, which includes one of two GXGXXG sites, is critical to DGK function. Although there is no evidence yet for catalytic activity, the homology indicates that p140 may be a new form of DGK which is under glucocorticoid regulation.

BZ 436 THE SERINE/THREONINE PROTEIN PHOSPHATASE INHIBITOR OKADAIC ACID PROMOTES OPENING OF VOLTAGE-DEPENDENT Ca^{2+} CHANNELS IN INSULIN-SECRETING CELLS.

Larsson, O., Haby, C*., Islam, M.S., Zwiller,J.* and Berggren, P-O. Department of Endocrinology, Karolinska Institutet, Box 60 500, 104 01 Stockholm, Sweden. *INSERM U338, 5 rue B. Pascal, 67084 Strasbourg, France.

Several reports suggest that the open probability of voltage-dependent Ca^{2+} channels is dependent on their state of phosphorylation. The purified L-type Ca^{2+} channel has been shown to be subject to phosphorylation by several protein kinases in vitro. It is known that voltage-dependent Ca^{2+} channels are inactivated by the Ca^{2+} ions entering through the channel during depolarization. One explanation for this phenomenon is dephosphorylation of the Ca^{2+} channel, probably through Ca^{2+} dependent phosphatases, and thereby reduced open probability. The effect of okadaic acid (OA), which blocks serine/threonine protein phosphatase type-1, -2A, -3, and to some extent type-2B, was studied with regard to Ca^{2+} movement through voltage-dependent Ca^{2+} channels in insulin-secreting cells. The free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) was studied by a microfluorometric method, applying Fura-2, and Ca^{2+} currents were investigated with the patch-clamp technique. Perifusing 1 μM OA for 1 minute caused a transient rise in $[Ca^{2+}]_i$. We also found a 40% increase in insulin secretion in the presence of 1 μM OA. The effect on $[Ca^{2+}]_i$ was dependent on the presence of extracellular Ca^{2+}. Interestingly, the OA-induced Ca^{2+} transient was totally abolished by the concomitant addition of the L-type Ca^{2+} channel D600 (50 μM). Stimulation of OA does not seem to affect $Ins(1,4,5)P_3$ induced mobilization of Ca^{2+}, since the OA evoked rise in $[Ca^{2+}]_i$ was unaffected by a previous addition of ATP (200 μM). Likewise, application of OA prior to a stimulation with ATP did not alter the ATP response. Furthermore, giving 1 μM OA to permeabilized RINm5F cells neither altered the uptake of Ca^{2+} to intracellular stores nor affected $Ins(1,4,5)P_3$-induced Ca^{2+} release, as measured with Ca^{2+}-selective electrodes. These results, thus, suggest that OA promotes Ca^{2+} entry into β-cells through voltage-dependent Ca^{2+} channels. Further support for a specific effect at the voltage-dependent Ca^{2+} channel is that 1 μM OA increased the Ca^{2+} current by 50% upon membrane depolarization from -80 mV to -20 mV. These data, thus, suggest that phosphatase activity has a profound effect in regulating the voltage dependence of the β-cell L-type Ca^{2+} channel.

BZ 437 K252A INDUCES TYROSINE PHOSPHORYLATION AND DIFFERENTIATION IN SY5Y CELLS INDEPENDENT OF PROTEIN KINASE-C INHIBITION, Anna C. Maroney, M. Elizabeth Forbes, Marcie A. Glicksman, Nicola Neff, Robert Siman and Craig A. Dionne, Cephalon Inc., 145 Brandywine Pkwy., West Chester, PA 19380

The protein kinase C (PKC) inhibitor K252a has been shown to promote cholinergic activity in rat spinal cord cultures and survival in chick dorsal root ganglion (Glicksman et al, submitted: Borasio, Neuroscience lett. 108:207-212, 1990). To determine the mechanism by which K252a behaves as a neurotrophic factor we examined the effects of this molecule in a human neuroblastoma cell line, SY5Y. In this cell line, K252a induces neurite outgrowth in a dose-dependent manner. One mechanism through which a large majority of growth factors transmit their signal is via tyrosine phosphorylation of a variety of substrates. To determine whether the growth factor-like effects of K252a are involved in this signaling pathway, the tyrosine phosphorylation profile of SY5Y lysates was examined. K252a induced tyrosine phosphorylation in a dose- and time-dependent manner. We observe a phosphorylation response to K252a administered in serum free medium by 30 min at a concentration of 100nM. The major phosphorylated proteins are of high molecular weight in the range of >p250, p180, and p130. These phosphorylation events are independent of PKC inhibition since its down regulation by long term incubation with phorbol esters does not block the effect of K252a. Similarly, the PKC inhibitors, H7 and calphostin, do not induce phosphorylation in the SY5Y cells; however staurosporine, a compound which is structurally related to K252a induces neurite outgrowth and selectively induces phosphorylation of the same high molecular weight proteins. A structurally related compound, K252b, which is not cell permeable, does not induce neurite outgrowth or the phosphorylation pattern. In PC12 cells, K252a does not induce neurite outgrowth or tyrosine phosphorylation. Tyrosine phosphorylation is also absent in non-neuronal cells such as fibroblasts treated with K252a. The K252a induced phosphorylation event is specific to responsive neuronal cells. The implication of the work done on primary cultures is that K252a may be a promising agent for the therapeutic application to spinal cord injury. The efficacy of K252a may be mediated by activating tyrosine phosphorylation of second messengers that are similarly modified by other growth factors.

BZ 438 PRESYNAPTIC NEUROTOXINS AS PROBES FOR PHOSPHOPROTEIN REGULATION OF ACETYLCHOLINE RELEASE FROM MAMMALIAN SYNAPTOSOMES, John L. Middlebrook and Dennis L. Leatherman, Toxinology Division, U.S. Army Medical Research Institute for Infectious Diseases, Frederick, MD 21701

Presynaptic neurotoxins are potent inhibitors of the depolarization-induced release of acetylcholine from presynaptic nerve terminals. Recent investigations have shown that the phosphorylation/dephosphorylation of certain presynaptic phosphoproteins may be involved in the mediation or modulation of neurotransmitter release. In the present investigation, rat brain synaptosomes were used to study the effects of several presynaptic neurotoxins on the phosphorylation of synaptic proteins. The toxins employed were the microbial toxin botulinum neurotoxin A (Botx) and two snake phospholipase A2 neurotoxins, taipoxin and textilotoxin. Cyclic nucleotide-(cAMP) dependent phosphorylation was potentiated two- to fivefold in synaptosomal lysates prepared from intact rat brain synaptosomes preincubated with taipoxin and textilotoxin. This effect was concentration dependent, although an enzymatically active, but non-neurotoxic phospholipase A2 from snake venom was approximately one hundredfold more potent than taipoxin or textilotoxin. In contrast, these two toxins elicited a 30-50% inhibition of calcium-dependent phosphorylation. The enhanced phosphorylation was observed in polypeptides with apparent molecular weights between 40 and 145 kD as determined by SDS-polyacrylamide gel electrophoresis. In contrast to the snake venom neurotoxins, the effects of Botx on phosphorylation were more selective. Phosphorylation of a 43kD protein was inhibited in both a concentration- and temperature-dependent manner. Effects on a lower molecular weight polypeptide were also noted. The relationships between toxins' effects on phosphorylation and inhibition of neurotransmitter release are under further evaluation.

BZ 439 PURIFICATION AND CHARACTERIZATION OF A PHOSPHATIDYLINOSITOL PHOSPHATE PHOSPHATASE FROM RAT BRAIN, Heidi M. Rath Hope and Linda J. Pike, Howard Hughes Medical Institute and the Department of Biochemitry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Mo. 63110

The availability of inositol phospholipids may be important in determining the responsiveness of a cell to agonists that signal via phosphatidylinositol turnover. The levels of these lipids are determined by their rates of synthesis and degradation. Our laboratory has previously purified and characterized a phosphatidylinositol 4-kinase from A431 cells. This enzyme phosphorylates phosphatidylinositol on the 4 position of the inositol ring. We are currently studying the enzyme responsible for the reverse reaction, a phosphatidylinositol phosphate phosphatase. Phosphatidylinositol phosphate phosphatase has been purified to greater than 2000- fold from rat brain. It initally partitions into the Triton X-100 insoluble pellet, but can be solubilized from that fraction by treatment with 1% Triton X-100 in the presence of 0.5 M NaCl. We have shown by lectin affinity and gel filtration chromatography that phosphatidylinositol phosphate phosphatase is a glycoprotein of approximately 70-80 kD. Glycan differentiation analysis of extensively purified phosphatidylinositol phosphate phosphatase suggests that it is modified by the addition of novel O-linked N-acetylglucosamine. The partially purified enzyme hydrolyzes both phosphatidylinositol 4-phosphate and phosphatidylinositol 3-phosphate, but does not utilize phosphatidylinositol 4,5-bisphosphate as a substrate. Based on estimates that the phosphatidylinositol phosphate phosphatase accounts for approximately 5% of the total protein in the purified preparation, it can be calculated that the specific activity of the homogeneous enzyme will be approximately 50 μmol/min/mg- a value similar to that seen for protein phosphatases and substantially higher than that of phosphatidylinositol 4-kinase.

BZ 440 **DECREASED PHOSPHORYLATION OF A LOW MOLECULAR WEIGHT SUBSTRATE OF cGMP-DEPENDENT PROTEIN KINASE IN NITROPRUSSIDE-RESISTANT HL-60 CELLS.** Jurgen S. Scheele, Renate B. Pilz and Gerry R. Boss. Departments of Medicine and Chemistry, University of California, San Diego, La Jolla, CA 92093-0652.

We have previously described the isolation of a variant subline of HL-60 cells that does not differentiate in response to the cGMP-elevating agent nitroprusside or to cGMP analogs. The data suggested that the defect was distal in the cGMP signal transduction pathway. We now show that these cells have normal cGMP-dependent protein kinase activity and by two-dimensional gel electrophoresis have identified 10 cGMP-dependent protein kinase substrates in the parental cells. Of these 10 proteins we found that one was phosphorylated to a considerably lesser extent in the variant than in the parental cells, both in vitro as well as in intact cells. In the parental cells this protein was phosphorylated within 15 min of adding nitroprusside suggesting it may serve a regulatory function during differentiation. This 23 kDa protein is a shared substrate of cAMP-dependent protein kinase and is not phosphorylated by this enzyme in the variant cells, either in vitro or in intact cells. Since cAMP analogs still induce differentiation of the variant cells, it appears that differentiation of HL-60 cells can be induced by phosphorylation of one of several proteins. Preliminary results by two-dimensional western-blotting suggest that the identified protein belongs to the ras superfamily.

BZ 441 BOVINE PARATHYROID CELLS EXPRESS PHOSPHOLIPASE C-α AND -γ_1, D. M. Shoback, T-H. Chen, B. Lattyak, Endocrine Research Unit, VA Medical Center, University of California, San Francisco, CA 94121.

Parathyroid hormone (PTH) secretion is regulated by the extracellular [Ca^{2+}]. Low [Ca^{2+}] maximally stimulates PTH release, and high [Ca^{2+}] inhibits secretion. The mechanism by which the [Ca^{2+}] is sensed by the parathyroid cell is unknown. It has been proposed that these cells express membrane sensors or receptors for Ca^{2+} that are coupled to phospholipase C (PLC) activation and sustained increases in intracellular free Ca^{2+} and 1,4,5-inositol trisphosphate (1,4,5-$InsP_3$). To determine which isoform of PLC might couple to the putative Ca^{2+} sensor in these cells, we extracted total and poly (A)$^+$ RNA from bovine parathyroid glands and performed Northern analysis using probes for PLC-α, β_1, β_2, γ_1, γ_2 and δ_1. We detected the hybridization of transcripts of ~2.0 and ~6.6 kB with labelled probes for PLC-α and PLC-γ_1, respectively. No hybridization was seen with β_1, β_2, γ_2, or δ_1 at low stringency. Since PLC-γ_1 is tyrosine phosphorylated by receptor tyrosine kinases in other systems, we examined whether raising extracellular [Ca^{2+}] stimulated the tyrosine phosphorylation of PLC-γ_1 in parathyroid cells. PLC-γ_1 was immunoprecipitated from whole-cell lysates using a rabbit antibody, electrophoresed on SDS PAGE gels, transferred to Immobilon, and blotted with anti-phosphotyrosine MAbs. Exposure of parathyroid cells to high Ca^{2+} (3 mM) for 5 to 30 minutes did not increase the tyrosine phosphorylation of PLC-γ_1, compared to control cells maintained at 0.5 mM extracellular Ca^{2+}. We conclude that raising extracellular Ca^{2+} and activation of the putative Ca^{2+} sensor in this cell does not likely involve tyrosine phosphorylation of PLC-γ_1. While PLC-γ_1 may not be the effector for the Ca^{2+} sensor in this system, receptors for other ligands, such as growth factors, may transduce signals using this PLC isoform in the parathyroid.

BZ 442EGF INDUCES A BIPHASIC PRODUCTION OF UNIQUE DIACYLGLYCEROL SPECIES FROM DIFFERENT PHOSPHOLIPID SOURCES:

Jianguo Song 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.*

Epidermal growth factor (EGF) induces increased levels of diacylglycerol (DG) that are rapidly detected when cells were prelabeled with [3-H]-arachidonic acid which is incorporated preferentially into phosphatidylinositol (PI). DG levels peak at 5 min after stimulation and then drop to background levels by 30 min and below background by 60 min. As the DG levels dropped, there was a corresponding increase in phosphatidic acid (PA) consistent with the conversion of DG to PA by a DG kinase. When cells were prelabeled with [3-H]-myristate, which is incorporated almost exclusively into phosphatidylcholine (PC), EGF also induced increased DG levels; however, DG levels peaked at 30 min, and remained elevated for greater than 2 hr. Thus, there appears to be a secondary response to EGF leading to the production of DG from PC. No significant increases in PA were observed in response to EGF when cells were prelabeled with [3-H]-myristate suggesting that the DG generated from PC is distinct from that derived from PI and is a poor substrate for DG kinase. Type D Phospholipase (PLD) activity, as measured by the transphosphatidylation of cellular phospholipids to phosphatidylethanol in the presence of exogenous ethanol, was also detected in response to EGF with kinetics that were similar to those observed for the DG produced from PC. Consistent with this hypothesis, increaseed PLD activity was only detected when cells were prelabeled with [3-H]-myristate; no PLD activity was observed when cells were prelabeled with [3-H]-arachidonic acid. These data suggest a biphasic production of DG in response to EGF that first involves the well characterized phospholipase C-mediated hydrolysis of PI-tris-phosphate to DG and inositol tris-phosphate, followed by a PLD-mediated hydrolysis of PC to choline and PA followed by the conversion of PA to DG by a PA phosphatase. These data also suggest that the different DGs produced from PI and PC are differentially metabolized and may also have different biological effects.

BZ 443 CHANGES IN INOSITOL 1,4,5-TRISPHOSPHATE AND SN 1,2-DIACYLGLYCEROL DURING MEIOSIS, MITOSIS AND FERTILIZATION IN XENOPUS LAEVIS. Bradley J. Stith, Biology Department, University of Colorado at Denver, Denver, CO 80217-3364

Using mass assays, we compare the changes in diacylglycerol (DAG) and inositol trisphosphate (IP3) during:

1. Hormone-induced meiotic cell division. With the use of insulin, insulin-like growth factor or progesterone, we note the hormones induce similar changes in IP3 and DAG levels before the cells enter prophase of meiosis.
2. Microinjection of ras p21 protein induces meiotic cell division but the pattern of IP3 and DAG changes before prophase is different from those produced by the three growth factors noted above.
3. We describe increases in DAG and IP3 after sperm addition to Xenopus eggs that are larger than those changes during the induction of meiosis.
4. During first mitotic division both DAG and IP3 were elevated.
5. During first cleavage (cytokinesis), DAG declined whereas IP3 increased.

Thus, using one cellular system, we report the first chronology of these second messengers through the induction of meiosis and during fertilization, mitosis and cytokinesis.

BZ 444 THE ROLE OF TYROSINE PHOSPHORYLATION IN PHOSPHOLIPASE D ACTIVATION AND IN PRIMING OF HUMAN NEUTROPHIL RESPONSIVENESS, Neil T. Thompson, Robert W. Bonser and Lawrence G. Garland, Wellcome Research Laboratories, Langley Court, Beckenham, Kent, BR3 3BS, UK.

Receptor-coupled phospholipase D (PLD) plays a major role in the function of human neutrophils. Activation of this enzyme by fMet-Leu-Phe is greatly enhanced by pretreating, or priming, the neutrophils with cytochalasin B (cyt B) and under these conditions is dependent on protein tyrosine phosphorylation (Uings *et al* 1992). Tyrosine phosphorylation appears to increase the efficiency of coupling between receptor and phospholipase, but the identities of the kinase and its substrate are unknown. Furthermore, the mechanism of action of cyt B and its relevance to more physiologically relevant priming is poorly understood. Therefore, we have assessed the activation of PLD in GMCSF- and TNFα-primed neutrophils and have investigated whether PLD is phosphorylated directly. A 30 min pretreatment of neutrophils with GMCSF or TNFα enhanced superoxide production in response to fMet-Leu-Phe and this was blocked by the tyrosine kinase inhibitor ST271 (IC_{50} = 0.6 μM). A 30 min pretreatment with GMCSF enhanced fMet-Leu-Phe-activated PLD by approx. 3-fold but this level of enhancement was only 40-50% of that observed in cyt B-primed cells. GMCSF induced tyrosine phosphorylation of several proteins within 30 min and PLD activation in GMCSF-primed cells was blocked by tyrosine kinase inhibitors. Anti-phosphotyrosine immunoprecipitates from cyt B- or GMCSF-primed cells did not contain a PLD activity measured using phosphatidyl[^{3}H]choline in a Triton X-100 micelle. Thus, tyrosine phosphorylation appears to be involved in the priming of neutrophil function by several agents. However, the role of tyrosine kinases in PLD activation is more complex than that now understood for PLCγ, in that it may not involve direct phosphorylation of PLD.

Uings, I.J., Thompson, N,T., Randall, R.W., Spacey, G.D., Bonser, R.W., Hudson, A.T. and Garland, L.G. (1992) Biochem. J. 281, 597-600

BZ 445GROWTH DEFECTS ASSOCIATED WITH DELETION OF THE PHO85 GENE OF *SACCHAROMYCES CEREVISIAE*, Barbara K. Timblin and Lawrence W. Bergman, Department of Microbiology and Immunology, Hahnemann University School of Medicine, Philadelphia, PA 19102

The PHO85 gene product is a protein kinase structurally related to the cdc2$^+$/CDC28 kinases and is required for the transcriptional repression of acid phosphatase (PHO5) production in yeast. Strains containing a PHO85 null allele exhibit multiple defects associated with growth of the cell in addition to constitutive expression of acid phosphatase. Strains lacking the PHO85 gene show reduced growth rate in medium containing glucose as a carbon source and fail to grow in medium containing glycerol/lactate. Interestingly, certain strains containing the deletion fail to grow in medium containing galactose as a carbon source due to the inability to induce one or more of the galactose-utilizing genes. This Gal$^-$ phenotype is not a general glucose-repression defect since expression of CYC1 and SUC2 are unaffected by the deletion of PHO85. Mating studies have shown that the galactose defect is due to a single, unlinked (to PHO85) gene present in these strains, termed PGL1 (Phosphate-Galactose Linker1). Using a plasmid library, we have cloned a gene, termed PGS1 (Phosphate-Galactose Supressor1), which in single copy allows the PHO85 deletion strain to grow in the presence of galactose; however, segregation analysis indicates that PGL1 and PGS1 are not allelic. When additional copies of PGS1 are present, suppression of the GAL$^-$ phenotype does not require the induction of the galactose-utilizing genes. Studies are underway to clone the PGL1 gene and to find the relationship between PGS1 and PHO85 in carbon metabolism and the cell's ability to grow.

BZ 446 PLC-γ ISOFORMS ARE TYROSINE PHOSPHORYLATED IN NATURAL KILLER CELLS DURING THE DEVELOPMENT OF BOTH DIRECT AND ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY. Adrian T. Ting, Larry M. Karnitz, Renee A. Schoon, Sofia Sarafova, Robert T. Abraham, and Paul J. Leibson, Dept. of Immunology, Mayo Clinic, Rochester, MN 55905

Natural killer (NK) cells are a subpopulation of lymphocytes with the capacity to kill malignant or virally-infected cells. This cytotoxic response can be initiated by: (1) the interaction of NK cell receptors with putative target structures on susceptible malignant or virally infected cells (direct NK cell-mediated cytotoxicity); or (2) the binding of low affinity receptors for IgG (FcγRIII) on NK cells to the Fc region of IgG-coated target cells (antibody-dependent cellular cytotoxicity). Either mode of stimulation results in the rapid activation of protein tyrosine kinase(s) and phospholipase C (PLC). PLC subsequently hydrolyzes membrane phosphoinositides, generating inositol-1,4,5-trisphosphate and *sn*-1,2-diacylglycerol as second messengers. Based on the paradigm provided by the receptor tyrosine kinases, we investigated whether PLC-γ1 and PLC-γ2 are expressed in NK cells, and whether these PLC isoforms are tyrosine phosphorylated in response to stimulation of NK cell surface receptors. Immunoblotting analyses with PLC-γ1 and PLC-γ2 specific antisera demonstrate that both isoforms are expressed in human NK cells. Furthermore, either binding to susceptible tumor targets or cross-linking of FcγRIII result in the tyrosine phosphorylation of both PLC-γ1 and PLC-γ2. These results suggest that receptor-induced phosphoinositide turnover in human NK cells is regulated by the tyrosine phosphorylation of PLC-γ isoforms. More broadly, these observations demonstrate that non-receptor protein tyrosine kinases activated by crosslinkage of a multi-subunit receptor can phosphorylate both PLC-γ isoforms.

BZ 447 PHOSPHATIDYLCHOLINE HYDROLYSIS AND c-*myc* EXPRESSION ARE IN COLLABORATING MITOGENIC PATHWAYS ACTIVATED BY COLONY-STIMULATING FACTOR 1. Xiang-Xi Xu, Teresa G. Tessner, Charles O. Rock and Suzanne Jackowski, Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38101

Mitogenesis and proliferation of the murine macrophage cell line BAC1.2F5 are initiated by the binding of colony-stimulating factor 1 (CSF-1) to its specific cell-surface receptor. The biochemical responses which follow include receptor tyrosine phosphorylation, activation of Ras, activation of serine/threonine kinases and immediate early expression of several genes such as c-*fos*, *junB*, and c-*myc*. Diglyceride production from hydrolysis of phosphatidylcholine (PC) by phospholipase C (PLC) is also within the first minutes and the maximum accumulation of diglyceride occurs at 15 min following CSF-1 addition to the cells. Addition of the PC-specific phospholipase C (PC-PLC) from *B. cereus* to the medium of quiescent BAC1.2F5 macrophages raises intracellular diglyceride and stimulates [^{3}H]thymidine incorporation to 33% of that evoked by CSF-1, although PC-PLC does not support continuous proliferation. PC-PLC treatment elicits only a subset of the biochemical responses associated with CSF-1 binding: PC-PLC stimulates expression of c-*fos* and *junB*, but not c-*myc* mRNA; PC-PLC does not activate CSF-1 receptor phosphorylation or degradation, GDP/GTP exchange on Ras, or Raf hyperphosphorylation. PC-PLC does not act through protein kinase C, since PC-PLC remains mitogenically active in cells chronically treated with phorbol esters, and in contrast to phorbol esters, does not activate Ras or Raf. PC-PLC treatment of BAC1.2F5 cells that constitutively express exogenous c-*myc* increases [^{3}H]thymidine incorporation to 86% of the level evoked by CSF-1 and sustains slow growth in the absence of growth factor. The data suggest that PC-PLC is a component of a signal transduction pathway that is downstream or independent of Ras activation and collaborates with c-*myc*. (supported by ACS BE-121B and GM45737)

Cell Cycles

BZ 500 REGULATION OF CELL CYCLE PROGRESSION AND THE NUCLEAR AFFINITY OF THE RETINOBLASTOMA PROTEIN BY PROTEIN PHOSPHATASES, Arthur S. Alberts[1], Andrew M. Thorburn[2], Jeff Frost[1], Shirish Shenolikar[3], Marc C. Mumby[4], and James R. Feramisco[2,5], [1]Biomedical Sciences Graduate Program, Departments of [5]Pharmacology and [2]Medicine, Cancer Center, University of California at San Diego, La Jolla CA 92093-0636; [3]Department of Pharmacology, Duke University Medical Center, Durham NC 27710; [4]Department of Pharmacology, University of Texas, Southwestern Medical Center at Dallas, Dallas TX 75235-9041

We examined the effects of microinjected serine/threonine specific protein phosphatases types 1 (PP1) and 2A (PP2A) on association of RB with the nucleus by testing the resistance of RB to extraction at the G1/S transition. Microinjection of PP1 into either the nucleus or cytoplasm of cells synchronized in G1 increased the amount of RB that was resistant to extraction from the nuclear compartment. Microinjection of PP2A, however, required direct injection into the nucleus to generate this effect. In addition, we found that the nuclear injection of only the catalytic subunit of PP2A (C) and not the complex containing the A and C subunits inhibited extraction of RB. For microinjection of either PP1 or PP2A, the resultant apparent increase in affinity of RB with the nucleus corresponded to inhibition of cell-cycle progression into S phase. Injection of either phosphatase into cells which had already entered S phase did not cause a block in DNA synthesis, suggesting that the effect of cell cycle arrest observed for the injected phosphatases was specific. Biochemical studies with purified PP1 and various isoforms of PP2A showed that intact RB protein phosphorylated with cdc2 kinase served as a substrate for both protein phosphatases *in vitro*. Furthermore, data from 2D-tryptic peptide mapping suggests that PP1 and PP2A preferentially dephosphorylate specific sites of RB protein phosphorylated *in vitro* by cdc2 kinase. Our results further suggest that protein phosphatases may be important in regulating the function of the RB protein and offer support for the idea that the ability of the RB protein to regulate cell cycle progression is dependent upon its phosphorylation state.

BZ 501 ROLE OF SIGNAL TRANSDUCTION KINASES/-PHOSPHATASES IN THE INDUCTION OF DNA SYNTHESIS IN SENESCENT PRIMARY FIBROBLASTS, David Alcorta, Cynthia Afshari and J. Carl Barrett, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

Fibroblast cultures of Syrian hamsters and humans enter a cell cycle arrest state known as cellular senescence after a specific number of cell doublings in culture. Stimulation of these cultures with serum or specific growth factors induces many but not all of the early response genes but does not to lead phosphorylation of the retinoblastoma gene (Rb) product, DNA synthesis, or additional rounds of cell division. Entry into the senescent state is accompanied by a down regulation of the G_1/S kinases, CDC2 and CDK2 and cyclin A mRNAs as well as a decrease in cyclin C, D_1 and E mRNAs (Afshari et.al. submitted). Further, an elevated phosphotyrosine content in pp44$^{MAPK/ERK1}$ relative to quiescent cells was found. The inhibition of DNA synthesis in senescent cells is overcome by stimulation with the tyrosine or serine/threonine specific phosphatase inhibitors, sodium orthovanadate or okadaic acid. In early passage Syrian hamster fibroblasts, this stimulation induces the synthesis of CDC2 protein, leads to RB phosphorylation and produces DNA synthesis (Afshari and Barrett, submitted). We are currently investigating the biochemical changes induced by these inhibitors in senescent cell populations. Specific interest is focussed upon the role of the early response kinases (pp70^{S6K}, pp90^{rsks}, and MEK), CDKs, cyclins, and serine/threonine and tyrosine phosphatases in the regulation of DNA synthesis in quiescent and senescent cells.

BZ 502 **THE REGULATION OF WEE1 KINASE,** Rosa M. Aligué and P. Russell, Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037

The timing of the onset of mitosis in fission yeast is primarily determined by the counteracting activities of the *wee1* and *cdc25* gene products. The Wee1 kinase acts as an inhibitor of mitosis by phosphorylating the Cdc2 kinase on tyrosine-15. The Cdc2 kinase is activated at the G2/M transition through the actions of the Cdc25 mitotic inducer, which is the protein tyrosine phosphatase that dephosphorylates Cdc2. Our studies are focussed on the regulation of the Wee1 kinase. Genetic and biochemical experiments have revealed that the formation of active Wee1 kinase requires the direct physical interaction with the product of a newly identified gene, *swo1*, a member of the hsp90 family. Hsp90 proteins are known to act as molecular chaperones for several protein kinases, including the *src* tyrosine kinase. We are exploring the possibility that Wee1 function is regulated by its interaction with Swo1. In a second avenue of investigation we have identified a small region of the Wee1 kinase, removed from the catalytic domain, that is critical for Wee1 mitotic inhibitor activity. Constructs lacking this region retain full kinase activity, and thus this region must be required in some way for substrate interaction in vivo. Our current experiments are aimed at identifying the function of this part of the Wee1 kinase.

BZ 503 GROWTH FACTORS INDEPENDENTLY REGULATE EXPRESSION OF CYCLINS D2 AND D3 DURING G1 PHASE IN THE HUMAN MYELOID CELL LINE MO7E.

K. Ando*, F. Ajchenbaum*, M. Hallek, and J.D. Griffin, Dana-Farber Cancer Institute, Boston, MA.

Hematopoietic cells respond to growth factors primarily during the G0 and G1 phases of the cell cycle. In yeasts and higher eukaryotes, there is abundant evidence that such external growth signals control the activities of a small number of cell cycle control proteins which regulate passage from G0 to G1 and from G1 to S. In virtually all cells, cyclins and cyclin-dependent kinases (cdk's) such as p34cdc2 are critically involved in controlling cell cycle progression. The recent identification of candidate G1 cyclins in mammalian cells has been a major advance in this field, but the exact functions of these cyclins (C, D1, D2, D3, and E) are unknown. The expression of the D cyclins was investigated in a GM-CSF/IL-3 dependent myeloid cell line, MO7e. After removal from growth factor for >12hours, the cells arrested in G0/G1 and did not express mRNA for either cyclin D1, D2, or D3. Treatment of starved cells with GM-CSF in the presence of FCS resulted in rapid induction of cyclin D2 RNA (early G1) and slower induction of cyclin D3 (late G1). Cyclin D1 was not detected in this cell line under any of several condidtions tested. In order to investigate the roles of these cyclins in growth control, cDNA's encoding both cyclin D2 and cyclin D3 were expressed as glutathione-S-transferase fusion proteins in bacteria, and these proteins were purified on affinity columns. Purified proteins were used to immunize rabbits to prepare specific antisera. Anti-cyclin D2 and D3 had minimal cross-reactivity, and also failed to react with cyclin A, C, or E when these proteins were produced by in vitro translation. MO7e cells were labelled with 35S-methionine, and cyclin D2 and D3 were immunoprecipitated at various times after stimulation with GM-CSF. In the absence of FCS, GM-CSF or SCF induced rapid (<2hr) expression of cyclin D2 protein, but not cyclin D3, and failed to induce entry of cells into S phase. In the presence of FCS, GM-CSF or SCF induced both cyclin D2 and D3, and induced entry into S phase.The effects of other factors on cyclin D2 and D3 induction are now under investigation. These results suggest that GM-CSF or SCF and serum provide distinct signals that are both required for traversing G1, and that result in differential regulation of two potentially key cell cycle control genes, cyclins D2 and D3. The induction of D3 may be a marker for a G1 restriction point in hematopoietic cells.

BZ 504 MITOSIS-SPECIFIC PHOSPHORYLATION OF POLYOMAVIRUS MIDDLE-T IS REQUIRED FOR CELL TRANSFORMATION, Kurt Ballmer-Hofer, Leonor Pérez, Friedrich Miescher-Institute, P. O. box 2543, CH 4002-Basel, Switzerland

Transformation of cells by polyomavirus is mediated by middle-T antigen, a protein able to form complexes with a cellular phosphatidylinositol-3-kinase, phosphatase 2A, and several members of the src family of tyrosine kinases ($pp60^{c\text{-}src}$, $pp62^{c\text{-}yes}$, and $pp59^{fyn}$). Middle-T has been shown to interfere with the regulation of $pp60^{c\text{-}src}$ during the cell cycle. In polyomavirus-transformed cells $pp60^{c\text{-}src}$ is constitutively dephosphorylated at tyrosine 527, a site negatively regulating the activity of this protein, while in normal cells this site seems to be only transiently dephosphorylated during mitosis. Consequently, middle-T-associated $pp60^{c\text{-}src}$ has high activity in both interphase and mitotic cells whereas the uncomplexed kinase is only transiently activated during mitosis. $pp60^{c\text{-}src}$ and middle-T are targets of a cell cycle regulated serine/threonine-specific kinase, $p34^{cdc2}$. Mutation of these phosphorylation sites in the c-src gene prevent mitosis-specific activation of the kinase and leads to a slight increase in the activity during interphase. In middle-T we identified two threonine residues in positions 160 and 291, respectively, that are targets of a cdc2-like kinase. Both aminoacids were mutated to alanine residues and the mutant proteins expressed in 3T3 cells. While the 291A mutant behaved as wild type, 160Amiddle-T and the double mutant, 160A·291Amiddle-T, were transformation-defective. Interestingly, the defective mutant proteins were still able to form all the complexes with cellular proteins known to date suggesting that additional characteristics of middle-T are required for cell transformation. The fact that middle-T and $pp60^{c\text{-}src}$ are phosphorylated differently in the various phases of the cell cycle suggests cell cycle-specific interactions with the regulatory machinery of the cell.

BZ 505 **CYCLIN A AND CDK2 SPECIFICALLY LOCALISE AT SUBNUCLEAR SITES OF DNA REPLICATION,**

M. Cristina Cardoso, Heinrich Leonhardt & Bernardo Nadal-Ginard, Howard Hughes Medical Institute and Department of Cardiology, Children's Hospital, Department of Cellular and Molecular Physiology and Department of Anatomy and Cellular Biology, Harvard Medical School, Boston, Massachusetts 02115

Terminally differentiated myotubes are permanently withdrawn from the cell cycle and do not synthesize DNA upon mitogen stimulation. We have previously developed a cellular system in which multinucleated myotubes can be induced to reenter the cell cycle by expression of SV40 large T antigen and inactivation of the retinoblastoma protein. Reversal of the terminally differentiated state is associated with re-induction of cyclin synthesis. Indirect immunofluorescence staining with anti-cyclin A antibodies of induced myotube nuclei revealed two different patterns. In addition to the formerly described disperse nuclear distribution, we have identified a distinct pattern of punctate and ring-shaped structures. We show that the latter corresponds to sites of DNA replication, by BrdU incorporation and costaining with BrdU-specific antibodies. Further analysis showed that also the cyclin A-associated cdk2 kinase is present at these sites. The association of cyclin A and cdk2 with DNA replication structures was also found in other cell types like ,e.g., mouse 3T3 fibroblasts, by using confocal laser scanning microscopy. This observation, therefore, seems to be valid for S phase in general. This subnuclear localization of the cyclin A and cdk2 kinase fits well with the recent observation by others that phosphorylation of RPA-32, within the initiation complex is required for DNA replication.

This novel finding provides useful informations on a link between cell cycle regulation and DNA replication. This approach of analyzing the subnuclear localization will be extended to other known cell cycle regulators in order to potentially sort out cell cycle regulatory signal transduction pathways.

BZ 506 PROTEIN PHOSPHORYLATION AND REORGANIZATION OF INTERMEDIATE FILAMENTS IN MITOTIC BHK CELLS. Ying-Hao Chou, Omar Skalli and Robert Goldman. Dept. of CMS Biology, Northwestern University, Chicago, IL 60611.

The extensive reorganization of interphase cytoskeletal networks is one of the hallmarks of M-phase cells. In BHK cells, the fibrous interphase network of intermediate filaments(IF) is rapidly transformed into protofilamentous aggregates as cells round up during the transition from interphase to mitosis. This structural remodeling is accompanied by the phosphorylation of both vimentin and the IF crosslinking protein, IFAP300. Biochemical fractionation and characterization of mitotic cell lysates has identified two protein kinases that are involved in the mitosis-specific phosphorylation of vimentin and IFAP300. One of these kinases is p34cdc2 and the other is correlated with a 38 kd protein. These two kinases phosphorylate vimentin at two mitotically unique sites which have been identified by directly sequencing of purified phosphopeptides. One of the sites resides in the non-alpha- helical N-terminus and the other in the C-terminal domain. In vitro assembly assays have been carried out to determine the effects of both kinases on vimentin polymerization. vimentin phosphorylated in the C-terminal domain forms only short IF while vimentin phosphorylated in the N-terminal domain fails to assemble into IF. It is also known that the phosphorylation and the disassembly of the nuclear IF proteins, the nuclear lamins, which accompanies nuclear envelope breakdown in M-phase, is mediated by p34cdc2. Taken together, the structural reorganization of both cytoplasmic and nuclear IF networks are major targets of mitosis-specific protein phosphorylation. (This work is supported by grants from NIGMS and NCI)

BZ 507 PHEROMONE-SPECIFIC MORPHOLOGICAL CHANGES AND GROWTH STIMULATION INDUCED BY *KSS1* IN *S. cerevisiae*, William E. Courchesne and Peter McNamara, Department of Microbiology, University of Nevada School of Medicine, Reno, NV 89557-0046

The simple eucaryote *Saccharomyces cerevisiae*, upon exposure to mating pheromone, arrests growth in the G1 phase of the cell cycle and undergoes a distinct morphological change. Cells with a temperature sensitive mutation in the *CDC28* gene (*cdc28^{ts}*) arrest cell cycle progression in G1 at the same arrest point caused by exposure to pheromones. Pheromone treatment of *cdc28^{ts}* cells incubated at the non-permissive temperature resulted in formation of multiple shmoo projections. Overexpression of the *KSS1* gene under the same conditions but without added phermones also induced shmoo-like projections, albiet greatly exaggerated. The ability of *KSS1* to induce the hyper-shmoo morphology was dependent on a functional pheromone-response pathway, and was suppressed by the pheromone-coupled G protein α subunit activated by mutation. The *KSS1*-induced morphology change was independent of microtubule function, and was instead correlated with an increase in the actin cytoskeleton. Moreover, in both exponentially growing cells and cell cycle arrested cells, *KSS1* overexpression stimulated overall cell growth, apparent as an increase in both cell size and protein content per cell. Thus, *KSS1* apparently is capable of stimulating both generalized cell growth and specialized cell growth associated with specific morphological changes.

BZ 508 CELL CYCLE-DEPENDENT NEGATIVE REGULATION OF p34^{cdc2} BY TRANSFORMING GROWTH FACTOR β1, Scott T. Eblen, Rebekah J. Burnette*, and Edward B. Leof*, Department of Cell Biology, Vanderbilt University, Nashville, TN 37232: and *Thoracic Disease Research, Mayo Clinic, Rochester, MN 55905.

Transforming Growth Factor β1 (TGFβ1) can either activate or suppress cellular growth in a cell-type specific manner. It has been established that TGFβ1 arrests quiescent restimulated epithelial cells in late G1 phase of the cell cycle. Inhibition of DNA synthesis occurred through a post-transcriptional action of TGFβ1. The mechanism of this growth arrest has not been conclusively established. Our primary studies have centered on the growth inhibitory effects of TGFβ1 in cycling CCL64 cells, a mink lung epithelial cell line. Release of sparse CCL64 cultures from TGFβ1 growth arrest also indicated a late G1 phase growth arrest. A potential regulator of mammalian cell cycle traverse is the serine-threonine protein kinase, p34^{cdc2}. Addition of TGFβ1 to cycling epithelial cells decreases the synthesis, phosphorylation, and kinase activity of p34^{cdc2} over a 24 hr period. The kinetics of the decrease in p34^{cdc2} synthesis precedes and parallels the decrease in DNA synthesis. TGFβ1 has no effect on either the steady state levels or turnover of p34^{cdc2}. The effects of TGFβ1 are cell cycle-dependent in that they are G1 specific. Moreover, this decrease in p34^{cdc2} synthesis by TGFβ1 is not simply reflecting a general decrease in protein synthesis since other growth regulatory proteins are not inhibited. A similar specific decrease of p34^{cdc2} synthesis in normal human mammary epithelial cells (HMEC) has been observed. Mimosine, a drug that also results in a late G1 block, arrests epithelial cells by a mechanism distinct from that induced by TGFβ1. Current studies comparing effects of mimosine and TGFβ1 on p34^{cdc2} and p33^{cdk2} will determine if inhibition of the protein(s) is a growth factor or growth regulatory event. Additionally, we are determining whether TGFβ1 is acting on the protein(s) through a transcriptional, post-transcriptional, or translational mechanism. Understanding the control of these important protein kinases may provide insight into the biochemical mechanism(s) of TGFβ1 growth inhibition.

BZ 509 **The Target of Rapamycin Defines a Restriction Point in Late G1** W. Michael Flanagan, Eduardo Firpo*, James M. Roberts*, and Gerald R. Crabtree. Howard Hughes Medical Institute. Unit in Molecular and Genetic Medicine. Beckman Center. Stanford University Medical School. Stanford, CA 94305. *Department of Basic Sciences. Fred Hutchinson Cancer Research Center. Seattle, WA 98104.

Rapamycin, a potent immunosuppressant and antifungal agent, inhibits an unknown evolutionarily conserved mechanism regulating S-phase entry. Delayed addition of rapamycin, up to 9 hours following interleukin 2 (IL-2) stimulation of T-cells, profoundly decreases proliferation suggesting that the mechanism of action of rapamycin is intimately involved in the mid/late G1->S phase of the cell-cycle. Rapamycin has no effect on the early G1 expression of c-myc, c-jun, cyclins D2, and D3. Remarkably, rapamycin completely inhibits the kinase activity of the cdk2-cyclin E complex despite normal expression of these proteins. Rapamycin also blocks the hyperphosphorylation of the retinoblastoma gene product (Rb), and prevents the induced expression of p34cdc2 and cyclin A. The action of rapamycin is antagonized by FK506 implicating an FKBP that can bind the transition-state mimic common to FK506 and rapamycin as the biologic receptor for these actions. Moreover, these data localize the site of action of rapamycin in late G1 and are consistent with a model in which cdk2-cyclin E phosphorylates pRb as a prelude to S-phase entry.

BZ 510 PHOSPHORYLATION INDEPENDENT ACTIVATION OF Cdk2 PROTEIN KINASE BY CYCLIN A.

Wade Harper, Lisa Connell-Crowley, Mark Solomon*, and Nan Wei; Department of Biochemistry, Baylor College of Medicine, Houston TX 77030 and * Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06510.

$p33^{Cdk2}$ is a serine-threonine protein kinase that associates with cyclins A and E and has been implicated in the control of the G1/S transition in mammalian cells. Recent evidence indicates that Cdk2, like Cdc2, requires cyclin binding and phosphorylation (of T160) for activation in vivo. However, the extent to which mechanistic details of the activation process are conserved between Cdc2 and Cdk2 is unknown. We have developed bacterial expression and purification systems for Cdk2 and cyclin A that allow mechanistic studies of the activation process to be performed in the absence of cell extracts. Recombinant Cdk2 is essentially inactive as a histone H1 kinase ($<4 \times 10^{-5}$ pmol phosphate transfered/min/μg Cdk2). However, in the presence of equimolar cyclin A, the specific activity is ~16 pmol/min/μg, 4×10^5-fold higher than Cdk2 alone. Mutation of T160 in Cdk2 to either alanine or glutamic acid had little impact on the specific activity of the Cdk2/cyclin A complex: the activity of $Cdk2^{T160E}$ was indistinguishable from Cdk2 while that of $Cdk2^{T160A}$ was reduced by 5-fold. To determine if the Cdk2/cyclin A complex could be activated further by phosphorylation of T160, complexes were treated with Cdc2 activating kinase (CAK), purified 12,000-fold from *Xenopus* eggs. This treatment resulted in 80-fold increase in specific activity (363 pmol/min/μg). This specific activity is comparable to that of the Cdc2/cyclin B complex after complete activation by CAK (1600 pmol/min/μg). Neither $Cdk2^{T160A}$/ cyclin A or $Cdk2^{T160E}$/cyclin A complexes were activated further by treatment with CAK. For Cdk2, both cyclin binding and phosphorylation contribute significantly to activation, although the energetic contribution of cyclin A binding is greater than that of T160 phosphorylation by ~5 kcal/mol. In contrast, the primary activation event for Cdc2 is T161 phosphorylation. Thus, while the specific activities and overall mechanism of activation are similar for Cdk2 and Cdc2, the respective contributions of cyclin binding and phosphorylation for Cdk2 activation are inverted for Cdc2.

BZ 511 CYCLINS A AND E AFFECT THE PHOSPHORYLATION STATE AND GROWTH SUPPRESSION CAPACITY OF THE RETINOBLASTOMA GENE PRODUCT

Lynn E. Horton, Yongyi Qian and Dennis J. Templeton, Institute of Pathology, Case Western Reserve University, Cleveland, OH 44106

The retinoblastoma protein (pRb) is multiply phosphorylated in a manner that varies throughout the cell cycle. The cell cycle kinases $p34^{cdc2}$ (or related proteins, possibly $p33^{cdk2}$) along with their regulatory subunits the cyclins are implicated in the phosphorylation of pRb. The activity of the G_1/S kinase-cyclin complexes coincide with the timing of pRb phosphorylation in the cell cycle. We have examined how the phosphorylation state and growth suppression characteristics of pRb are influenced by $p34^{cdc2}$, $p33^{cdk2}$ and cyclins *in vivo*. Co-expression of cyclins A or E with pRb in Saos-2 cells show that these cyclins affect the phosphorylation pattern of pRb, increasing the amounts of the hyperphosphorylated (inactive) form. Co-expression of cyclin B does not affect the phosphorylation pattern of pRb. Phosphorylation of pRb is enhanced by co-expression of cyclins A or E with either $p34^{cdc2}$ or $p33^{cdk2}$, but is not affected when the kinases are co-expressed alone with pRb.

Cyclins A and E also counteract the growth suppression ability of pRb, since co-expression of these cyclins (but not cyclin B) with pRb into Saos-2 cells inhibits the cell growth suppression manifested by pRb. Co-expression of cyclin D_1 with pRb results in a small change in the phosphorylation pattern of pRb, but cyclin D_1 has no effect on the growth inhibition activity of pRb. We conclude that cyclins A and E are able to influence phosphorylation of pRb, and that this correlates with the ability of these cyclins to inhibit the growth controlling activities of pRb. Cyclins A and E are therefore likely to contribute to the cell cycle regulation of pRb.

BZ 512 BIPHASIC ACTIVATION OF TWO MITOGEN-ACTIVATED PROTEIN KINASES DURING THE CELL CYCLE IN MAMMALIAN CELLS,

Takashi Kadowaki, Hiroyuki Tamemoto, Kazuyuki Tobe, Kojiro Ueki, Michio Kohno, Masato Kasuga, Yasuo Akanuma, and Yoshio Yazaki, Tokyo University School of Medicine, Tokyo 113, Japan.

Recently mitogen-activated protein kinase (MAPK) in the *Xenopus oocyte* was shown to be activated in the M phase. MAPKs in the fibroblasts of the mammals are activated when quiescent cells are stimulated with a variety of growth factors and are believed to be involved in the G_0 to G_1 transition. In this study, we addressed whether MAPKs of mammals are controlled in a cell-cycle dependent manner. We studied mitogen-activated protein kinase (MAPK) activities during the cell cycle of Chinese hamster ovary (CHO) cells using site-specific antibodies against extracellular signal-regulated kinase-1, a 44-kDa MAPK (Boulton, T.G., Yancopoulos, G.D., Gregory, J.S., Slauer, C., Moomaw, C., Hsu, J., and Cobb, M.H. (1990) Science 249, 64-67). These antibodies detected two distinct MAPKs (44- and 42-kDa MAPKs) in CHO cells. CHO cells were arrested at metaphase in the M phase by treatment with nocodazole, and activities of MAPKs were analyzed at specific time points after release from arrest. Immune complex kinase assay and renaturation and phosphorylation assay in substrate-containing gel revealed that both 44- and 42-kDa MAPKs had activities in the G_1 through S and G_2/M phases and were activated biphasically, in the G_1 phase and around the M phase. MAPKs were inactivated in metaphase-arrested cells. The amount of MAPKs did not change significantly in the cell cycle. The phosphorylation state of the MAPKs was analyzed by immunogrecipitation of MAPKs from cells labeled with 32p *in vivo* followed by two-dimensional gel electrophoresis and autoradiography. In the G_1, S and G_2/M phases, MAPKs were phosphorylated on both tyrosine and threonine residues and dephosphorylated in metaphase-arrested cells. Our data suggest that MAPKs may play some role in the cell cycle other than G_0/G_1 transition.

BZ 513 LOVASTATIN, AN INHIBITOR OF HMG-COA REDUCTASE, CAN BLOCK BOTH S-PHASE ENTRY AND EXIT FROM G2/M IN MITOGEN-ACTIVATED HUMAN PERIPHERAL BLOOD LYMPHOCYTES,

W.K. Kang, M.J. Ha, C.E. Myers, and J.B. Trepel, Clinical Pharmacology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, 20892

Following mitogenic stimulation peripheral blood lymphocytes undergo a series of tightly regulated events that move this population from G_0 into the cell cycle and through cell division. Lovastatin, which has been used clinically to lower cholesterol synthesis has been shown to reversibly synchronize a variety of tumor cells and normal cells in the G_1 phase of the cell cycle. HMG-CoA reductase is the rate-limiting enzyme in the cholesterol pathway. A variety of intermediates in this pathway as well as the end-product cholesterol have the potential for playing a critical role in growth regulation. To better understand the control of cell cycle progression in normal and neoplastic cells and to examine the potential mechanism of lovastatin-induced cell synchronization we have studied the effect of lovastatin on prominent events in T cell activation and cell cycle progression.When peripheral blood lymphocytes were stimulated with 2 μg/ml phytohemagglutinin (PHA) by day 1 they moved from G_0 into G_1, as determined by induction of IL-2 receptor expression, by day 2 transferrin receptors were expressed and the cells had moved into S phase, as determined by ^{3}H-thymidine incorporation and flow cytometric analysis, and by day 3 DNA histograms showed a normal log phase distribution. When lovastatin was added prior to or simultaneously with PHA the cells were blocked in G_1 as has been reported. Unexpectedly, when lovastatin was added 12 hours after PHA and the cells were studied for the next 3 days, DNA histograms showed 2 prominent peaks without an intervening S-phase. Thus lovastatin can induce both a block to entry into S phase and a block to exit from G_2/M. To begin to dissect the mechanism of lovastatin action we examined the expression of 2 proteins known to be important in cell cycle control; the retinoblastoma protein (RB), which, in its unphosphorylated form is thought to block entry into S phase, and cyclin B, the degradation of which is thought to be required for cells to exit G2/M. As determined by Western blot analysis, lovastatin markedly down-regulated the amount of RB protein, with the greatest decline occuring in the phosphorylated form. In contrast, lovastatin blocked the normal cell cycle-dependent loss of cyclin B. These data suggest new mechanisms for lovastatin-induced cell cycle synchronization and demonstrate unexpected connections between the HMG-CoA reductase pathway and critical cell cycle components.

BZ 514 UPREGULATION OF c-myc INDUCES cdc2 AND cdk2 EXPRESSION, Young Ho Kim, Meredith A. Buchholz, Francis J. Chrest and Albert A. Nordin, Gerontology Research Center, 4940 Eastern Avenue, Baltimore, MD 21224

The expression and/or upregulation of several early T-cell activation genes requires the presence of IL-2 well before the traditional IL-2 regulated entry of the cells into S phase. Murine G_0 T cells activated by immobilized anti-CD3 in the absence of IL-2 failed to express cdc2, to sustain the expression of IL-2Rα and TfR and to upregulate the constitutively expressed cdk2, c-myc, and IL-2Rß. When IL-2 was provided exogenously during the activation period, the normal pattern of gene expression was restored. This, together with the observation that the addition of anti-IL-2 and IL-2R during the activation period induced the same aberrant pattern of gene expression indicated that the interaction of IL-2 with its high affinity receptors during early G_1 regulated the expression of several genes which are necessary for the cells to traverse G_1 and enter S phase. Reversibly blocking the activated T-cells in late G_1 with [2-(4 hydroxytoluene-3-yl)-4,5-dihydro-4 carboxythiazole] (HTDCT; Hoechst 768159) had no effect on the expression of TfR, IL-2Rα or IL-2Rß. However there was no upregulation of c-myc or cdk2 and no expression of cdc2. The addition of rIL-2 during activation in the presence of HTDCT did not restore the normal pattern of gene expression. The presence of antisense c-myc oligonucleotide during the activation of G_0 T cells inhibited the expression of cdc2 and cdk2 without affecting the expression of IL2-R or l8S rRNA and the cells accumulated in late G_1. These studies demonstrate that c-myc expression regulates the expression of the cdks and suggest a role for c-myc in the G_1/S transition.

BZ 515 ABROGATION OF A KINASE MEDIATED G1 CELL CYCLE ARREST POINT IS A LATE EVENT IN THE NEOPLASTIC PROGRESSION OF HUMAN FIBROBLASTS TRANSFECTED WITH THE SV40 LARGE T ANTIGEN GENE. Paul M. Kraemer and E. Morton Bradbury, Life Sciences Division, Los Alamos National Laboratory, Los Alamos, NM 87545

Cell cycle arrest points that are sensitive to the kinase inhibitor staurosporine have been shown to have widely differing sensitivities for processes in G1 and G2. In addition, the exquisitely sensitive G1 arrest point has been reported to be abrogated in neoplastically transformed cells. Using a multi-step model of the neoplastic process in human cells, we show here that abrogation of this arrest point occurred in five of eleven tumorigenic cell populations. The abrogation, in those instances when it occurred, was a late step and associated with the acquisition of tumorigenicity, but apparently independent of conventional criteria for in vitro transformation. The data also show that reversible sequestration of the drug complicates the potential usefulness of staurosporine for therapeutic purposes. Research supported by the U.S. Department of Energy.

BZ 516 THE *Doa* LOCUS ENCODES THE *DROSOPHILA* HOMOLOGUE OF MAMMALIAN *CDC2-LIKE KINASE*, Kun Lee, Bokyoung Yun, Robert Farkas, and Leonard Rabinow, Waksman Institute, Rutgers University, Piscataway, N.J. 08855-0759.

We are studying the structure and function of the *Darkener of apricot* (*Doa*), locus of *Drosophila*, which was identified in mutagenic screens for dosage-sensitive modifiers of gene expression. *Doa* mutants display a maternal effect, are recessive lethal, increase transcription of the copia retrotransposon and alter the expression of a number of host genes. By sequencing cDNA clones, we found that *Doa* encodes the *Drosophila* homologue of murine and human *cdc2-like protein kinase* (*clk*), with 49% overall amino-acid **identity**. *clk* kinase is structurally related to serine/threonine kinases, but auto-phosphorylates at tyrosine residues as well. Other data shows that a DNA-binding activity in wild-type nuclear extracts specific for a fragment of the copia LTR is eliminated by *Doa* mutations. This DNA-binding activity is also eliminated by treatment of wild-type extracts with acid phosphatase, suggesting that the protein responsible must be phosphorylated to bind DNA, and that it is directly or indirectly a substrate of *Doa* kinase. Finally, we've also found that photoreceptor cells degenerate in the most extreme *Doa* alleles, accompanied by disorganization in the lamina, implicating *Doa* in the regulation of one or more neural-specific genes.

BZ 517 CYCLIN E DISPLAYS PROPERTIES APPROPRIATE FOR A G1 CYCLIN. Lees, E[1]., Tsai, L.H[1]., Faha, B.F[1]., Riabowol, K[2]. and E.Harlow[1]. [1] MGH Cancer Center, Building 149, 13th St, Charlestown MA 02129. [2] University of Calgary, Calgary, Canada.

Progression through the cell cycle is tightly controlled. The proteins that give rise to this coordinate regulation include the well characterised $p34^{cdc2}$ kinase and the more recently isolated cdk family. Regulating the activity of these kinases is another diverse but related family of cyclins, whose periodic expression apparently gives rise to the temporal pattern of kinase activity seen through the cell cycle. To understand further how this control is brought about necessitates the detailed examination of such cyclin/cdk complexes.

We have raised antibodies to one of the recently isolated cyclins, cyclin E, and used these to examine the contribution of this cyclin to the overall control of the cell cycle. We have shown that cyclin E binds to $p33^{cdk2}$, with associated kinase activity that peaks in G1. Microinjection and depletion experiments imply an absolute requirement for this kinase for commencement of DNA synthesis. We are now trying to identify appropriate substrates for this kinase, that might facilitate control at the G1/S boundary. One potential substrate is the transcription factor E2F, which associates with cyclin E in a cell cycle dependent manner. E2F has been shown to be important in the control of several genes required for DNA synthesis, and it is therefore attractive to speculate that a cyclin E associated kinase might regulate the transcriptional complex containing E2F during G1 to regulate entry into S phase. Other substrates include the adenovirus E1A associated proteins p107 and p130. We have shown similarly that these proteins only act as substrates during the early stages of the cell cycle.

Our recent experiments show that cyclin E is targetted by the adenovirus E1A protein in an analogous fashion to cyclin A. We believe that E1A operates by association with important regulatory proteins to disrupt normal control of cellular division, allowing cells to prematurely enter DNA synthesis where the virus can then replicate its own DNA. Cyclin E would be an attractive target for the virus as a controller of entry into S phase.

BZ 518 REGULATION OF A DNA DAMAGE RESPONSE IN S-PHASE, Vivian Liu and David Weaver, Dana-Farber Cancer Institute and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA 02115

When normal human cells are exposed to DNA damaging agents such as ionizing radiation, initiation of DNA replication is primarily inhibited, presumably to allow for DNA repair before the damaged regions are replicated. Thus, S-phase is temporarily disrupted. Cells from ataxia telangiectasia (AT) patients do not exhibit these effects when exposed to ionizing radiation. The inhibition of cellular DNA synthesis after exposure to ionizing radiation is delayed in AT cells, the extent of inhibition is less than that of wild-type cells, and the recovery to near normal levels of DNA synthesis is more rapid in AT cells. AT is a chromosomal breakage syndrome characterized by a variable immunodeficiency, and a predisposition to malignancy.

We have used the SV40 replicon as a model system to examine the regulation of S-phase in wild-type and AT cells. We have shown that radioresistant DNA synthesis of SV40 DNA replication occurs in AT cell but not in wild-type cells. We will present data suggesting the existence of an X-ray-inducible trans-acting factor responsible for inhibiting DNA synthesis. Secondly, we are examining the role of phosphorylation and desphosphorylation of DNA replication factors in this DNA damage response. We will also present evidence that RPA, one of the 7 cellular proteins required for SV40 DNA replication, is phosphorylated in response to X-rays. Din et al., (1990) have shown that as cells enter S-phase, the p34 subunit of RFA becomes phosphorylated. These experiments may suggest that phosphorylation and/or dephosphorylation of cellular proteins regulate DNA damage responses in the cell cycle.

BZ 519 ISOLATION AND CHARACTERIZATION OF RECOMBINANT PROTEIN KINASE C MEMBERS PKC -δ, -ε, -η AND -ζ. Marek Liyanage, Anna Kapsokefalou, David Frith and Silvia Stabel, Max-Delbrück-Laboratorium in der Max-Planck-Gesellschaft, Carl-von-Linné-Weg 10, D-5000 Köln 30, Germany

In order to compare the regulatory and catalytic properties of the novel protein kinase C members we expressed PKC-δ, -ε, -ζ and PKC-η as recombinant proteins from their cDNAs in mammalian COS cells and in insect cells via baculovirus vectors (1). Although PKC-δ, -ε and -η show similar phorbol ester binding activities as conventional PKC enzymes, they show a distinctively different behaviour towards conventional PKC substrates like histone, myelin basic protein, protamine or protamine sulphate, suggesting, that either phorbol esters are not able to fully activate these enzymes or that their substrate specificities are quite different from those of the conventional PKC enzymes.

Using a simple purification procedure we have purified recombinant PKC-ζ. This enzyme does not bind phorbol ester and is not activated by phorbol ester or several diacylglycerols on any substrate tested. Protamine sulfate and a synthetic peptide derived from the pseudosubstrate sequence serve as good substrates for PKC-ζ, however, phosphorylation is independent of classic PKC activators.

Site-directed mutagenesis of the zinc finger domains of conventional PKCs and PKC-ζ is used to identify residues which are crucial for phorbol ester binding.

Results will be discussed in view of the different behaviour of these enzymes in phorbol ester binding.

(1) Liyanage, M., Frith, D., Livneh, E. & Stabel, S. Biochem J. 283, 781-787 (1992)

BZ 520 THE CYCLIN-DEPENDENT KINASES OF HUMAN T LYMPHOCYTES. Joseph J. Lucas, Agota Szepesi, Attila Tordai, Joanne Domenico, Naohiro Terada and Erwin W. Gelfand. National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado, 80206.

The regulation of expression of the genes encoding the PSTAIRE sequence-containing, cyclin-dependent kinases was investigated in normal human T lymphocytes purified from peripheral blood. Resting G0 phase cells were stimulated to proliferate in vitro by treatment with a combination of a phorbol ester(PDB) and ionomycin. Resting cells expressed, at a low level, the gene encoding the p33(cdk2) kinase and a single protein species was detected by immunoblotting. After stimulation, this protein increased in amount and was joined by a second immunoreactive species with an increased mobility upon gel electrophoresis. The latter species was first detected at about 15 hrs after PDB/ionomycin treatment, i.e., in mid-G1 phase, about 12-15 hrs before the initiation of DNA replication. The cdc2 gene, in contrast, was not expressed in resting cells; its protein products, three differentially phosphorylated species, were first detected in activated T cells just prior to S-phase entry. H1 histone-kinase activity was also assessed in p13(suc) precipitates and in specific immunoprecipitates containing either p33(cdk2) or p34(cdc2). Resting cells contained a very low level of activity which increased in amount in mid- G1 phase, corresponding to the apparent activation of p33(cdk2). H1 histone kinase activity due to p34(cdc2) was detectable in S phase but was most pronounced later in the cell cycle, as cells entered mitosis. In proliferating cells it appeared that in amount and activity, p33(cdk2) was comparable to that of p34(cdc2), but that the two enzymes showed complex differential patterns of regulation during the cell cycle. Finally, it was noted that low levels of H1 histone kinase activity could also be detected in immunoprecipitates prepared using an antibody directed to the product of the cdk3 gene, an observation now under further investigation. The role(s) of the cyclin dependent kinases in early cell cycle-related events, such as the initial phosphorylation of the product of the Rb gene and transduction of the signals initiated by IL2/IL2-receptor interaction, will be discussed.

BZ 521 CYCLIN B BUT NOT CDC25 IS REQUIRED FOR THE G2-SPECIFIC ACTIVATION OF THE NIMA PROTEIN KINASE OF ASPERGILLUS NIDULANS, Sarah Lea McGuire and Stephen A. Osmani, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030 and Weis Center for Research, Geisinger Clinic, Danville, PA 17822

The nimA gene of Aspergillus nidulans encodes a protein kinase which is required for the G2-M transition of the cell cycle and which has been proposed to function in parallel to the $p34^{cdc2}$ histone H1 kinase (Osmani et al., 1991, Cell 67:283). We have examined the cell cycle-specific regulation of NIMA and found that it is regulated post-transcriptionally. Although slight changes in nimA mRNA levels are observed during the cell cycle, these do not account for observed changes in NIMA activity and NIMA protein abundance. Both NIMA activity and NIMA protein levels are low during G1/S, increase greatly at G2, reach a peak at M, then decrease dramatically as cells exit mitosis and enter G1 of the next cell cycle. As both cyclin B and the cdc25 tyrosine phosphatase are required for the activation of $p34^{cdc2}$, we have studied the potential role of these proteins in the G2-specific activation of NIMA. Results indicate that mutation of $nimE^{cyclinB}$ arrests cells in G2 with low NIMA kinase activity and low NIMA protein abundance, hence preventing the G2 activation of NIMA. A G2 arrest imposed by mutation of $nimT^{cdc25}$ does not affect this G2 activation, as full NIMA activity and high NIMA protein levels are observed during this arrest. These data suggest that cdc25 is required for the activation of $p34^{cdc2}$ alone, while cyclin B activates not only $p34^{cdc2}$ but also NIMA. Cyclin B is thus a common element of the NIMA and $p34^{cdc2}$ protein kinase pathways.

BZ 522 RAPAMYCIN-INDUCED INHIBITION OF $p34^{cdc2}$ KINASE ACTIVATION IS ASSOCIATED WITH G_1-S-PHASE GROWTH ARREST IN T LYMPHOCYTES, William G. Morice, Gregory J. Brunn, Gregory Wiederrecht*, John Siekierka*, and Robert T. Abraham, Dept. of Immunology, Mayo Clinic, Rochester, MN 55905 and *Dept. of Immunology, Merck Sharp and Dohme Research Laboratories, Rahway, NJ 07065

The immunosuppressive macrolide, rapamycin (RAP), blocks cytokine-dependent T-cell proliferation through binding to a family of intracellular receptors termed FKBPs. Although the actual mechanism is unknown, current models suggest that the RAP-FKBP complex interferes with a signaling event critical for cytokine-stimulated cell-cycle progression. In this study, we demonstrate that RAP inhibits IL-2-dependent growth of the murine T cell line, CTLL-2, by blocking cell-cycle progression at or near the G_1-S-phase boundary. Furthermore, IL2-induced expression of the early response genes, c-*myc* and c-*jun*, was insensitive to RAP, suggesting that the drug's inhibitory effects were localized to a more distal event in G_1. Previous studies of the yeast cell cycle demonstrated a critical role for activation of the serine-threonine kinase $p34^{cdc2}$ in both the G_1-S and G_2-M-phase transitions. In CTLL-2 cells, we observed that IL-2 stimulation caused an abrupt increase in $p34^{cdc2}$ histone-H1 kinase activity which immediately preceded S-phase entry. The activated form of $p34^{cdc2}$ kinase appeared in a high molecular weight complex (M,,275,000) which was resolved from the monomeric, inactive kinase (M,,34,000) by gel-filtration chromatography. The formation of the high molecular weight complex and the accompanying increase in $p34^{cdc2}$-specific histone-H1 kinase activity were inhibited by treatment of the cells with RAP. These results indicate that RAP suppresses IL-2-dependent T-cell growth by disrupting an event required for commitment of late G_1-phase cells to enter S-phase. Moreover, the signaling pathway leading to the formation of the activated $p34^{cdc2}$ kinase may be the critical target for the RAP-FKBP complex in IL-2-stimulated T-cells.

BZ 523 INHIBITORS OF EUKARYOTIC CELL CYCLE FROM MICROBIAL METABOLITES, **Hiroyuki Osada[1, 2], Fumio Hanaoka[2] and Kiyoshi Isono[3], [1]Antibiotics, [2]Biodesign Research, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, [3]Tokai Univ., Shimizu, Shizuoka 424, Japan**

In the past few years, we have come to realize that protein kinases and phosphatases play an important role for controlling the eukaryotic cell cycle. We have isolated several new inhibitors of protein kinases and phosphatases from microbial metabolites which inhibited the cell cycle progression at G2 or M phase.

The staurosporine analogues, RK-286C and RK-1409B were isolated as inhibitors of protein kinases. The inhibitors were found to cause DNA re-replication of mammalian cells without mitosis, resulting in the production of polyploid cells.

We have also discovered a new protein phosphatase inhibitor, tautomycin, which inhibited the cell cycle progression of a mouse temperature sensitive mutant, tsFT210 strain at M phase. tsFT210 cells defective in $p34^{cdc2}$ were arrested in G2 phase by the culture at 39.4°C and the cells were released from the arrest by shifting down to 32°C. When the cells were simultaneously released from the G2 arrest and exposed to tautomycin, the cell cycle progression was stop at M phase.

The results obtained using microbial inhibitors enable us to clarify the sequential events in the cell cycle progression, especially in M phase.

BZ 524 CELL CYCLE CONTROL MECHANISMS IN MOS TRANSFORMED CELLS, Nelson Rhodes[1], Cynthia Innes[1], Ray Hicks[2], Aisha Kasenally[3], Friedrich Propst[3] and Richard S. Paules[1], [1]Mammalian Molecular Genetics Group, NIEHS, RTP, NC 27709, [2]ICRF, 91 Riding House Street, London W2 1PG, U.K., [3]Ludwig Institute for Cancer Research, Saint Mary's Hospital Medical School, Norfolk Place, London W2 1PG, U.K.

The mos oncogene (v-mos) is the transforming gene of the Moloney murine sarcoma virus (Mo-MSV) encoding a serine/threonine protein kinase. Expression of v-mos or the mouse cellular homolog (c-Mos) from a Mo-MSV LTR efficiently transforms NIH/3T3 cells. The mos protein associates with $p34^{cdc2}$ and has been shown to phosphorylate tubulin, vimentin, and cyclin B in vitro. Although c-mos has been demonstrated to have an essential function controlling the G2-M transition in oocytes, recent reports suggest that a G1 function of mos is a prerequisite for its transforming activity.

We have been investigating cell cycle control in mouse cells overexpressing v-mos and c-Mos. Following serum deprivation, $p34^{cdc2}$ is barely detectable in nontransformed cells, whereas mos transformed cells have dramatically increased levels of the $p34^{cdc2}$ protein. Moreover, we find that the $p34^{cdc2}$ protein is present in various phosphorylated forms and has significant histone H1 kinase activity. These serum starved cells have a G0/G1 DNA content when analyzed by flow cytometry, and are not synthesizing DNA. Upon serum stimulation, we detect a synchronous increase in DNA synthesis indicating that the cells were arrested in either G0 or early G1. We are currently investigating whether these increased levels of $p34^{cdc2}$ are due to the increased expression of $p34^{cdc2}$ message or a result of increased protein stability and what proteins, if any, are associated with this kinase-active $p34^{cdc2}$. These increased levels and kinase catalytic activity of $p34^{cdc2}$ in mos transformed cells versus nontransformed cells during serum starvation may have implications regarding the mechanism of mos induced transformation.

BZ 525 REGULATION AND FUNCTION OF MURINE CDC25B, Byron Sebastian and Tony Hunter, Molecular Biology and Virology Laboratory, The Salk Institute for Biological Sciences, P.O. Box 85800, San Diego, CA, 92186

Three mammalian homologs of the fission yeast *cdc25* mitotic inducer have been identified. Cdc25 family member(s) activate the cdc2/cyclin B serine/threonine kinase by dephosphorylating cdc2 on threonine-14 (T-14) and tyrosine-15 (Y-15). CDK2 is a cdc2-like kinase which, when complexed with cyclin A or cyclin E, is believed to regulate cell cycle progression through S-phase. We have found that CDK2 is also phosphorylated on T-14 and Y-15, and that treatment of cyclin A, cyclin E, or CDK2 immunoprecipitates with purified bacterially expressed Cdc25M2 (the murine homolog of human CDC25B) increased the histone H1 kinase activity of these immunoprecipitates 5-10 fold. In each case, activation occurred concomitant with the specific dephosphorylation of CDK2 on T-14 and Y-15. Our results suggest that the phosphorylation and dephosphorylation of CDKs on T-14 and Y-15 may regulate not only G2/M but also other cell-cycle transitions, and raise the possibility that each CDK may be regulated by one or more Cdc25 family member(s). We have raised antisera specific for Cdc25M2 and are further investigating the substrate specificity and regulation of Cdc25M2 in an attempt to understand its role in the cell cycle.

Phosphorylation of Transcription Factors

BZ 526 THE REGULATION OF TRANSCRIPTION BY THE RETINOBLASTOMA SUSCEPTIBILITY GENE PRODUCT AND CYCLINS, Jalila Adnane[1], Zhaohui Shao[1], Seong-Jin Kim[2] and Paul D. Robbins[1], [1]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261 and [2]Laboratory of Chemoprevention, National Cancer Institute, Bethesda, Maryland 20892

The protein product of the retinoblastoma tumor suppressor gene (Rb) negatively and positively regulates transcription of certain promoters in a transient cotransfection assay. We have identified several *trans*-acting factors that are able to mediate transcriptional regulation by Rb. In particular, we have demonstrated that Rb can stimulate transcription mediated by the Sp1 and ATF-2 transcription factors. More recently, we have used a GAL4-Myc fusion protein to demonstrate that Rb can positively regulate Myc-mediated transcription in 3T3 and CCL-64 cells. Furthermore, we have mapped the domains in Myc and Rb responsible for the observed Rb-mediated regulation of Myc activity *in vivo* to the domains previously shown to be responsible for the interaction between Rb and Myc *in vitro*. These results suggest that Rb can positively regulate transcription mediated by at least three transcription factors, ATF-2, Sp1, and Myc, through protein-protein interaction.

It has been demonstrated previously that cyclin A and Rb can interact independently with the E2F transcription factor and that a cyclin A-cdk2 complex can phosphorylate Rb. To determine the role of cyclin A as well as other cyclins in regulating transcription, we have examined the ability of cyclins to regulate transcription from the adenovirus E2 promoter as well as GAL4-Sp1-mediated transcription in a transient cotransfection assay. We have demonstrated that human cyclin A, human cyclin D1 (PRAD1), and both mouse cyclin D1 and D3 can stimulate E2 promoter activity in 3T3 and CCL-64 cells. The stimulation of E2 promoter activity by these cyclins is mediated through both the ATF and E2F binding sites. In contrast, cyclin A slightly stimulates GAL4-Sp1-mediated transcription whereas mouse and human cyclin D1 strongly repress GAL4-Sp1-mediated transcription. These results suggest that certain cyclins can differentially regulate the activity of specific transcription factors, possibly through a Rb-dependent pathway. To examine further the function of cyclins and cyclin-cdc2-like complexes on transcription and on the activity of specific transcription factors, we have fused cyclins A, B, C, D1, D2, D3, and E as well as Rb and p107, to the GAL4 DNA binding domain. In preliminary experiments, we have observed significant affects of specific GAL4-cyclin fusion proteins as well as GAL4-Rb on transcription, dependent upon a GAL4 binding site in the chimeric promoter reporter plasmids.

BZ 527 DISSECTING SIGNALLING PATHWAYS THAT LEAD TO THE PHOSPHORYLATION OF TRANSCRIPTION FACTORS, Sadhana Agarwal, Nidhi G. Williams, Helene Paradis and Thomas M. Roberts, Dana Farber Cancer Institute and Harvard Medical School, Boston, MA 02115

Several cytoplasmic proteins including the non-receptor tyrosine kinase pp60 ^{src}, the small G-protein p21^{ras} and the serine-threonine kinases Raf-1 and MAP kinase (p44^{erk1}) have been shown to play critical roles in the transmission of activating signals from the cell surface to the nucleus. We have been using the baculovirus/Sf9 cell system to study the effect of these proteins on each other and to try to unravel signalling cascades that may connect them. Using coexpression of different combinations of these proteins in Sf9 cells, we find that the kinase activity of p44^{erk1} is increased by coexpression with p21$^{v\text{-}ras}$ but only to a limited extent. This activation of p44^{erk1} by p21$^{v\text{-}ras}$ is greatly enhanced by coexpression with Raf-1 suggesting that at least one pathway for activation of p44^{erk1} by p21$^{v\text{-}ras}$ occurs via Raf-1. Consistent with this hypothesis, a truncated, activated form of Raf-1 alone is sufficient to activate p44^{erk1} to a level similar to that seen by p21$^{v\text{-}ras}$ and Raf-1 together. We are now using this system to examine potential downsteam targets of these activated kinases. Results from experiments examining the effects of such upstream signalling cascades on the phosphorylation of the transcription factor c-jun will be presented.

BZ 528 PHOSPHORYLATION OF HUMAN PROGESTERONE RECEPTOR: MODULATION OF RECEPTOR FUNCTION BY ACTIVATORS OF CELLULAR KINASES AND PHOSPHORYLATION BY ERKS *IN VITRO* , C.A. Beck, A. Poletti, Y. Zhang, S.K. Nordeen, N.L. Weigel, D.P. Edwards, Pathology , Univ. of Colorado HSC, Denver, CO 80262 and Cell Biology, Baylor College of Medicine, Houston, TX. 77030

Human progesterone receptors (hPR) are ligand-inducible transcriptional regulators. In response to hormone binding , hPR become hyperphosphorylated suggesting a link between phosphorylation and receptor activation. Recent studies have shown that modulators of cellular kinases and phosphatases enhance PR-dependent transcriptional activity when PR are bound to agonists. In the present study, we tested the effects of various modulators of protein phosphorylation on the activity of PR when bound to the progestin antagonists RU486 or ZK98299. Stimulation of cyclic nucleotide dependent protein kinases with 8-Br cAMP resulted in the RU486 induction of PR -mediated transcription of a reporter gene (MMTV-CAT) stably transfected in human breast cancer cells (T47D) and of an endogenous progesterone responsive gene, human metallothionein. In the absence of 8-Br cAMP, RU486-PR complexes are inactive. Unlike RU486, the progestin antagonist ZK98299 does not induce PR-DNA binding and does not exhibit transcriptional activity in the presence of 8-BR cAMP. Thus activation of signal trans-duction pathways can alter the biologic activity of PR when bound to antagonists (i.e. RU486) that promote receptor-DNA binding. Phosphotryptic peptide mapping has shown that PR-B and PR-A are multiply phosphorylated and that increased phosphorylation in response to hormone binding occurs in at least two stages, a very rapid phosphorylation and a slower phosphorylation that is associated with a decrease in mobility of PR on SDS-gels. This raises the possibility that different protein kinases are responsible for each phosphorylation and could serve different functions. A computer search of hPR revealed the presence of a MAP (mitogen activated protein) kinase consensus sequence (PXS/TP) located in the N-terminus of the B isoform of hPR. MAP kinase is a member of the family of extracellular signal regulated kinases (Erks). Highly purified preparations of hPR are efficiently phosphorylated by MAP kinase *in vitro* and as determined by phosphotryptic peptide mapping, MAP kinase phosphorylates several physiological sites that are also phosphorylated on PR *in vivo*. Thus a potential candidate enzyme for PR phosphorylation has been identified.

BZ 529 INVOLVEMENT OF PROTEIN KINASES IN THE IL_6 STIMULATION OF TYPE II PHOSPHOLIPASE A_2 GENE TRANSCRIPTION. Gilbert Bérézíat, Lan Kong, and Jean-Luc Olivier. CNRS research Unit on lipoproteins and phospholipases CHU SAINT ANTOINE, PARIS VI UNIVERSITY, FRANCE.

Type II phospholipase A_2 (PLA_2) has been implicated in both the general inflammatory response and local reactions. Type II PLA_2 gene expression seems to be under the control of cytokines in a tissue dependent manner.

In the liver, synthesis and secretion of type II PLA_2 is stimulated by interleukine 6 (IL_6), interleukine 1 (IL_1) and tumor necrosis factor (TNFα). We have identified an acute phase respensive element (APRE) in the proximal promotor sequence of type II PLA_2 gene and demonstrated that it binds several C/EBP related proteins from rat liver nuclear extracts. Some of them are induced in rat liver after lipopolysaccharide (LPS) treatment.

CAT assays performed in $HepG_2$ cells with deleted mutants allowed us to demonstrate that type II PLA_2 APRE is responsable for most of the IL6 effect on gene transcription in this cell type. The effect of IL_6 on the CAT activity of $HepG_2$ cells transfected with a minimal PLA_2 promoter-CAT construct, was mimicked by either forskolin or dibufyryl cyclic AMP. In contrast to their effect on the endogenous gene, IL_1 was found ineffective and TNFα inhibited both basal and IL_6 stimulated CAT activity in transfected cells. This indicates that IL_1 and TNFα effects on the endogenous PLA2 gene occur at a post-trancriptional level. The effect of TNFα was mimicked by the tetradecanoyl phorbol acetate (TPA) a potent activator of protein kinase C. In addition, maximal phosphorylation by okadaic acid, a potent inhibitor of protein phosphotases blocked both basal and IL_6-stimulated CAT activities.

Our results suggest that, in $HepG_2$ cells, IL_6-stimulated type II PLA_2 gene transcription is under the regulatory control of C/EBP-related proteins whose efficiency might be positively and negatively modulated by protein kinases.

BZ 530 ENHANCED CAMP LEVELS INDUCE RAPID DEPHOSPHORYLATION OF RB

Heidi Kiil Blomhoff[1], Trond Stokke[2], Kristin B. Andersson[1], Erlend B. Smeland[1], Jon Christoffersen[1],
[1]Dept. of Immunology, [2]Dept. of Biophysics, Institute for Cancer Research, The Norwegian Radium Hospital, 0310 OSLO, Norway

Enhanced levels of cAMP enforced by forskolin inhibits the proliferation of the B-lymphoid cell-line Reh. The cells very rapidly become arrested in the mid G1 phase of the cell cycle, and transiently also in the G2 phase. Associated with the G1 block, the RB protein becomes dephosphorylated. After 4 hours of forskolin treatment a shift in the phosphorylation pattern is noted on Western blots, and the number of RB positive G1 nuclei increases from approximately 10% to 87%. As the cells are transiently blocked in the G2 phase for the first 10 hours of forskolin treatment, the increased number of RB positive G1 nuclei appears to be due to active dephosphorylation of previously phosphorylated RB rather than accumulation of cells in the RB positive "window" in early G1. The dephosphorylation of RB is prevented by okadaic acid, indicating that a phosphatase is involved in the process. Associated with the cAMP-induced dephosphorylation of RB, the MYC level becomes downregulated at the RNA as well as at the protein level. The reduced MYC level is due to inhibition of transcriptional initiation. We are currently investigating whether dephosphorylation of RB could be involved in the reduced transcription of MYC through sequestration of the MYC transcription factor E2F.

BZ 531 PHOSPHORYLATION OF JUND AND ITS POSSIBLE ROLE IN IMMEDIATE EARLY GENE EXPRESSION

Libert H.K. Defize, Hubrecht Laboratory for Developmental Biology, Utrecht, The Netherlands.

JunD, in contrast to its molecular relatives, cJun and JunB, is present in detectable amounts in resting NIH-3T3 cells, both on the RNA as well as on the protein level. We have investigated the possible significance of this phenomenon. We show that: i) Upon TPA treatment of the cells, there is a 20-50 fold increase in phosphorylation of two Serine residues in the N-terminus of JunD, while phosphorylation of its DNA-binding domain is relatively unaffected; ii) Mutation of these Serine residues to Aspartic acid greatly increases the transactivation potential of JunD in transient transfection assays; iii) The specificity of JunD in activating reporter constructs containing different TRE sequences is different from cJun; iv) Knocking out JunD in transient transfection assays using anti-sense expression constructs impairs immediate early gene transcription. These data indicate that JunD is a primary target for the signal transduction cascade initiated by activation of protein kinase C.

We are currently investigating the effect of stably transfecting anti-sense JunD into various cell-types. The results will be discussed.

BZ 532 HEAT SHOCK ACTIVATES A PROTEIN KINASE AND A PROTEIN PHOSPHATASE ACTING ON THE C-TERMINAL DOMAIN OF RNA POLYMERASE II LARGEST SUBUNIT

Marie-Françoise Dubois, Aniko Venetianer*, Van Trung Nguyen and Olivier Bensaude, Department of Biology, Ecole Normale Supérieure, 46 Rue d'Ulm, 75005 Paris, France. *Institute of Genetics, Szeged, Hungary.

At normal growth temperature, the RNA polymerase II largest subunit of eukaryotic cells is distributed between the non-phosphorylated IIa form and the highly phosphorylated IIo form. We found that a stress, such as a heat-shock, induced a shift from the IIa form towards the IIo form in human and in rat cells. In contrast, in murine cells the enzyme was dephosphorylated. However, when these cells were submitted to a heat-shock in the presence of a phosphatase inhibitor such as okadaic acid, the RNA polymerase II CTD was heavily phosphorylated.

The stress activation of a CTD-kinase was postulated, since lysates from heat-shocked mammalian cells exhibited a strong activation of a protein-kinase which phosphorylated a synthetic peptide consisting of four repetitions of the CTD motif (hepta-4) and since the CTD of a β-galactosidase/CTD fusion protein was increasingly phosphorylated *in vivo* upon heat-shock.

In human and in rat cells, the stress activated CTD-kinase was more potent than the stress activated phosphatase, while in murine cells, the CTD-phosphatase counteracted the CTD-kinase. The concomitant activation of a CTD-kinase and a CTD-phosphatase may contribute to ease transcription initiation and entry into elongation during stress conditions.

BZ 533 PROGRAMMED CELL DEATH: A POSSIBLE ROLE FOR PROTEIN PHOSPHATASES AND KINASES

Duygu Findik, Glenn Baxter, Qizhong Song, Martin Lavin, Queensland Institute of Medical Research, 300 Herston Road, Brisbane, QLD, Australia, 4029.

Apoptosis or programmed cell death is a distinct type of cell death and results from induction of active processes within the cell. Apoptosis can be defined morphologically by distinct characteristic changes. In murine thymocytes there is a requirement for RNA and protein synthesis for apoptosis to occur. However, the majority of human cell types appear to undergo apoptosis independent of RNA and protein synthesis. Protein modification especially phosphorylation/ dephosphorylation may play an important role in the mechanism of apoptosis in human cells. We have shown that apoptosis caused by heat treatment and ionising radiation in the lymphoma and leukemia cell lines is accompanied by the dephosphorylation of a few specific proteins. One of these proteins is common to both cell lines. Okadaic acid an inhibitor of phosphatases - 1 and 2A prevented apoptosis in all cases and inhibited the dephosphorylation of this common protein as well as several others (Baxter and Lavin, 1992; Song et al.,1992). In order to study the role of phosphatases in apoptosis we employed sense and antisense oligonucleotides for PP-1 and PP-2A. Antisense oligonucleotides for PP-1 protected cells from undergoing apoptosis. However, neither antisense nor sense oligonucleotides for PP-2A had any effect on apoptosis. We have also employed H-89 and Calphostin C, specific inhibitors of cAMP dependent protein kinase (PKA) and protein kinase C (PKC) respectively to investigate the role of protein kinases in apoptosis. Inhibition of PKA increased the level of apoptosis from 18% to 31% in a B cell line treated with ionising radiation whereas inhibition of PKC did not have any effect on apoptosis.

1- Baxter, G. and Lavin, M.F. J. Immunol. 148, 1949-54, 1992
2- Song, Q. et al. J. Cell. Physiol. (in press).

BZ 534 THE ROLE OF AP-1 IN V-SRC INDUCED MITOGENESIS IN CHICKEN EMBRYONIC FIBROBLASTS M.C.Frame, J.A.Wyke and A.D.Catling. The Beatson Institute for Cancer Research, Garscube Estate, Switchback Road, Bearsden, Glasgow G61 1BD.

Activation of v-Src in chicken embryo fibroblasts (CEF) made quiescent by serum deprivation results in both morphological transformation and transition from G_0 to G_1 and on to cell division. We have investigated early changes in endogenous AP-1 regulation in response to mitogenesis induced by activation of a temperatue sensitive mutant of v-Src (RCAN-LA29) or addition of serum. Reactivation of the membrane associated tyrosine kinase results in a several fold increase in AP-1 DNA binding and similar increase in activity of an AP-1 responsive reporter. In quiescent RCAN-29 infected CEF cultures stimulated into cycle by shift to permissive temperature, c-fos transcripts are elevated by 15 minutes and remain considerably above basal levels for at least 4 hours. In response to adding serum, the stimulation in c-fos transcripts is much greater although transient, peaking at 30 minutes and declining to basal levels by 1 hour. Despite the difference in magnitude of the transcript response, the stimulation of nuclear c-Fos protein is similar in both serum and v-Src stimulated cultures. No difference in c-jun transcripts or nuclear c-Jun protein level is detected on activation of v-Src. However, there is an early difference in the tryptic phosphopeptide map of p39 c-Jun in response to both v-Src and serum. On stimulation there is a redistribution of phosphate in the C-terminal phosphopeptides which may be responsible in part for the increase in AP-1 DNA binding. Phosphorylation of N-terminal serines 63 and 73 on peptides X and Y, known to be responsible for regulation of the transactivation function of c-Jun, is constitutively high in resting CEF cultures and neither serum nor v-Src results in altered phosphorylation at these sites. Thus, in stationary CEF cultures activation of v-Src or addition of serum results in an early increase in AP-1 activity which appears to be mediated by stimulation of c-Fos and altered phosphorylation at the C-terminus of c-Jun. Reactivation of a myristylation defective version of the RCAN-29 v-Src protein is unable to induce resting CEF cultures to re-enter cycle and is non-transforming. In addition, this mutant fails to induce early increases in AP-1 activity implying that these nuclear changes require crucial signalling events at the membrane and correlate with the biological consequences of activating the v-Src tyrosine kinase.

BZ 535 REGULATION OF TERNARY COMPLEX FORMATION ON THE *c-fos* PROMOTER BY PHOSPHORYLATION Hendrik Gille and Peter E. Shaw, Max-Planck-Institut für Immunbiologie, Spemann Laboratories, Stübeweg 51, Postfach 1169, D-7800 Freiburg, Germany.

The expression of the proto-oncogene *c-fos* is rapidly and transiently induced by a variety of extracellular signals. Trancriptional activation by most mitogens is mediated through the SRE, which is bound by the transcription factors SRF and $p62^{TCF}$. Formation of this ternary complex has been shown to correlate with inducibility of the *c-fos* gene by serum, TPA and EGF *in vivo*. The mitogen-activated protein (MAP) kinases, ERK1 and ERK2, are also rapidly induced by mitogens. Their activity can be detected in nuclei within 5 min. after stimulation of cells.

Ternary complex formation with the *c-fos* SRE is stimulated upon phosphorylation of $p62^{TCF}$ by ERK2 *in vitro*. Enhanced ternary complex activity is also detected in cells under conditions that selectively activate ERK1 and ERK2.

Two independently isolated cDNA clones, Elk-1 and SAP-1, encode proteins that have the SRF-dependent DNA-binding characteristic of $p62^{TCF}$. Elk-1 is phosphorylated by both ERK1 and ERK2 on C-terminal threonine residues. We are currently mapping the phosphorylation sites and investigating the effects of phosphorylation on DNA binding by Elk-1.

BZ 536 THE DUAL EFFECT OF ADENOVIRUS E1A ON THE TRANSCRIPTION FACTOR cJUN. Bertine Hagmeyer, Peter Angel*, Hans van Dam, Monique Duyndam, Harald König*, Ingrid Herr*, Alt Zantema, Peter Herrlich* and Alex van der Eb. Lab. for Molecular Carcinogenesis, University of Leiden, Leiden, The Netherlands. * Institut für Genetik und Toxikologie, Kernforschungszentrum Karlsruhe, Karlsruhe, Germany.

In cells, transformed by the adenovirus E1A gene, the collagenase gene is downregulated and the c-jun gene is upregulated. These effects were shown to be mediated by TPA-responsive elements (TRE's) present in the promoter regions of the affected genes. Both TRE sequences are recognized by cJun-containing dimeric complexes. In order to determine the mechanism by which E1A downregulates the collagenase TRE we tested chimeric constructs in which either the DNA binding domain (DBD) or the transactivation domain (TD) of cJun was replaced by the corresponding part of the transcription factor GHF1. The repression of cJun activity by E1A turned out to be directed towards the DBD of cJun, whereas the TD was actually stimulated by E1A. These data were supported by in vivo footprinting experiments, showing that in E1A-expressing cells the collagenase TRE is not occupied. This indicates that E1A represses the binding of cJun/cJun homodimers and cJun/cFos heterodimers, the complexes that normally bind to the collagenase TRE. On the other hand, binding to the c-jun-TRE was not affected. Recently, we have shown that the activation of the c-jun-TRE by E1A is mediated via the cJUN/ATF-2 heterodimer. Since the DNA binding activity of cJun has been reported to be regulated by phosphorylation, we investigated whether E1A interferes with cJun DNA binding by altering the phosphorylation status of the DBD. Sofar we don't have evidence that indeed this is the case. We have, however, detected hyperphosphorylation of the TD of cJun in adeno-transformed cells, which is very similar to the activating hyperphosphorylations of cJun induced by e.g. Ras and TPA. This phenomenon is consistent with our observed activation of the TD of cJun by E1A. Our main interest is now to unravel the mechanism by which some cJun-containing dimers (cJun/cJun and cJun/Fos) are selectively inactivated by E1A, whereas others, e.g. cJun/ATF-2, are not inactivated, or even activated.

BZ 537 PHEROMONE-DEPENDENT PHOSPHORYLATION OF STE12, Wesley Hung and Ivan Sadowski, Department of Biochemistry, University of British Columbia, Vancouver, B.C. V6T 1Z3

Binding of mating pheromones to cell surface receptors of *Saccharomyces cerevisiae* activates a signal transduction pathway, known as the pheromone response pathway, which results in increased transcription of genes required for mating. Some components of this pathway have been identified, which include the protein kinases STE7, STE11, FUS3 and KSS1. Induction of pheromone response genes requires the transcriptional activator STE12 , which binds to a specific DNA sequence called the pheromone response element in promoters of pheromone inducible genes. STE12 is hyperphosphorylated in response to pheromone. We have found that pheromone-dependent phosphorylation of STE12, and a fusion between the GAL4 DNA binding domain and STE12 activation domain, requires the protein kinases of the pheromone response pathway. Mutation of the zinc requiring DNA binding motif of the GAL4-STE12 fusion abolishes DNA binding but not pheromone-dependent phosphorylation, suggesting that pheromone-dependent phosphorylation of STE12 is not a consequence of its activity as a transcriptional activator. In addition, a STE12 mutant containing internal deletion of the DNA binding domain is not phsophorylated in response to pheromone and is present mainly in the cytoplasm, whereas wild-type STE12 is nuclear localized even prior to pheromone treatment. We conclude from these results that pheromone-dependent phosphorylation of STE12 requires all the kinases of the pheromone response pathway and proper subcellular localization to the nucleus. Moreover, the STE12 kinase must be present in, or translocated to the nucleus in response to pheromone.

BZ 538 Inflammatory cytokine gene expression requires synergy between multiple phosphorylation pathways and defined regulatory proteins - Steven G. Irving and Yassamin Jalinoos, Dept. of Pathology, Georgetown University School of Medicine, Washington, DC 20007

huMIP-1b is a cytokine which exhibits proinflammatory and myelopoietic properties, and is expressed in response to mitogenic stimulation of human T lymphocytes and various nonproliferative stimuli to other hematopoietic cells. Using a limited region of the huMIP-1b promoter, we have recapitulated the characteristic signaling requirements of the endogenous gene and have localized them to two adjacent cis elements that act in a cooperative fashion to confer mitogen inducible transcriptional responsiveness to minimal promoter constructs. Interestingly, for a number of other genes in a wide array of cell types, each of these elements, a CRE (cAMP response element) and an NF-kB-like element, is known to act autonomously in mediating inducible expression in response to characteristic phosphorylation signals. Despite the dependence for inducible expression on synergy between two "ubiquitous" regulatory elements in the huMIP-1b promoter, the expression of huMIP-1b is restricted to hematopoietic cells. Transfection of non-expressing cells (low passage human fibroblasts) with a truncated huMIP-1b promoter containing only the CRE and NF-kB-like sites only minimally relieves the restricted expression, although this promoter retains inducibility to high levels in human Jurkat T cells. We are analyzing the huMIP-1b promoter to examine whether tissue restricted expression results from the unique configuration of widely distributed regulatory elements, the recognition of these by tissue specific variants of more widely expressed regulatory factors, and the potential for additional elements which limit expression in a dominant and tissue restricted fashion.

BZ 539 ADENOVIRUS E4ORF4 PROTEIN ASSOCIATES WITH PHOSPHATASE 2A AND CONTROLS PHOSPHORYLATION AND GENE EXPRESSION IN ADENOVIRUS-INFECTED CELLS, Tamar Kleinberger, Ulrich Müller, and Thomas Shenk, Department of Molecular Biology, Howard Hughes Medical Institute, Princeton University, Princeton, NJ 08544

Adenovirus E1A protein and cAMP cooperate to induce transcription factor AP-1 and viral gene expression in mouse S49 cells. We report now that a protein encoded within the viral E4 gene region acts to counterbalance the induction of AP-1 DNA-binding activity by E1A and cAMP. Studies with mutant adenoviruses demonstrate that in the absence of E4orf4 protein, AP-1 DNA-binding activity is induced to substantially higher levels than in wild-type virus-infected cells. The induction is the result of increased levels of junB transcription and an elevated production of junB and c-fos proteins. Furthermore, hyperphosphorylated forms of c-fos and E1A proteins accumulate in the absence of functional E4orf4 protein. We propose that E4orf4 induces phosphorylation-dependent alterations in the activity of c-fos, E1A or some as yet unidentified protein, which may then lead to decreased synthesis of AP-1 components.

To understand the mechanisms by which the viral protein influences both phosphorylation and gene expression, we have isolated cellular proteins which associate with E4orf4. One of these proteins has been identified as phosphatase 2A, and its B subunit mediates the interaction. We are currently studying the effect of E4orf4 on phosphatase 2A activity and the mechanisms by which the phosphatase mediates the E4orf4 effects on gene expression.

The E4orf4 function likely plays an important role in natural infections since a mutant virus unable to express the E4orf4 protein is considerably more cytotoxic than the wild-type virus.

BZ 540 THE HTLV-I TAX$_1$ PROTEIN ASSOCIATES WITH PROTEIN KINASE C, Paul F. Lindholm, Robert L. Reid, Fatah Kashanchi, and John N. Brady. Laboratory of Molecular Virology, NCI, Bethesda, MD 20892.

HTLV-I infection is associated with adult T-cell leukemia and tropical spastic paraparesis. HTLV-I infected lymphocytes show increased expression of NF-kB regulated cellular genes including IL2-R$_\alpha$, TNF-$_\beta$ and IL-6. Expression of the Tax$_1$ gene product has previously been shown to be associated with induction of the NF-kB transcription factor. We have previously shown that purified, recombinant Tax$_1$ protein added to the tissue culture medium is taken up by uninfected cells and transported to cytoplasmic and nuclear compartments. Cellular Tax$_1$ uptake was followed by a rapid and transient increase in NF-kB DNA binding in 70Z/3 lymphocytes. The induction of NF-kB did not require de novo protein synthesis and did not lead to the induction of NF-kB p105 or c-rel mRNA. Therefore we considered the possibility that Tax$_1$ induction of NF-kB may occur by a cytoplasmic activation mechanism leading to the release of the IkB inhibitor from sequestered NF-kB complexes. NF-kB DNA binding has been regulated in vitro by phosphorylation of IkB in NF-kB complexes and alteration of the oxidation state of NF-kB proteins. We now demonstrate that Tax$_1$ protein associates with protein kinase C (PKC) by in vitro immunoprecipitation. The addition of Tax$_1$ to the reaction leads to the phoshorylation of Tax$_1$ and a 10-fold increase in the autophosphorylation of PKC in the absence of phosphatidylserine and diolein. Maximal Tax$_1$-PKC activation occured under reducing conditions. The role of the Tax$_1$-PKC interaction and phosphorylation in the regulation of the NF-kB transcription factor will be discussed.

BZ 541 THE CYTOSKELETON AS A TARGET AND AS A MODULATOR OF TRANSCRIPTIONAL REGULATION AND PHOSPHORYLATION-BASED SIGNAL TRANSDUCTION: REGULATION OF CHOLINERGIC RECEPTOR EXPRESSION AND FUNCTION, Ronald J. Lukas and Merouane Bencherif, Division of Neurobiology, Barrow Neurological Institute, Phoenix, AZ 85013

Cytoskeletal elements maintain cell shape and motility and actively participate in mitosis, process outgrowth, and intracellular trafficking. Clearly, these 'structural' elements are dynamic targets of signaling cascades that affect many aspects of cell function, and their numbers are subject to transcriptional control. Here we present data suggesting that actin microfilament networks may also 'function' to mediate transcriptional effects and to modulate intracellular signaling.

Disruption of actin networks in TE671/RD cells with cytochalasin B induces dose- and time-dependent increases in numbers of nicotinic acetylcholine receptors (nAChR; ^{125}I-labeled alpha-bungarotoxin binding sites) in cellular membrane fractions. There is no change in cell surface nAChR numbers or function (measured using a $^{86}Rb^+$ efflux assay) on drug treatment, indicating that nAChR upregulation is confined to an intracellular pool and is not a response to drug-induced loss of nAChR function. Cytochalasin effects on nAChR numbers are similar to, but not additive with, upregulatory effects of nicotine treatment, but are inhibited by simultaneous treatment of cells with phorbol-12-myristate-13-acetate. Cytochalasin exposure induces a coordinate, 3-4-fold increase in levels of mRNA (Northern analysis) encoding each of the four nAChR subunits expressed by TE671/RD cells. By contrast, cytochalasin treatment has no effect on numbers (^{3}H-labeled QNB binding sites) of muscarinic acetylcholine receptors (mAChR), but enhances mAChR function (carbachol-stimulated accumulation of inositol phosphates).

These studies suggest that actin microfilament networks may play roles in transcriptional regulation of integral membrane receptor expression and in modulation of receptor-mediated, kinase-targeted signaling cascades. A hypothetical model is proposed that views the cytoskeleton as a functioning link between the plasma membrane and the nucleus and as a substrate for intracellular cross-talk.

BZ 542 IDENTIFICATION AND CLONING OF DNA SEQUENCES THAT BIND AND ACTIVATE THE DNA-DEPENDENT PROTEIN KINASE (DNA-PK). Nusrat Parveen Malik, Vladimir Poltoratsky, Ivana Vancurova, and Timothy H. Carter, Dept. of Biological Sciences, St. John's University, Jamaica, NY 11439

Highly purified preparations of DNA-PK form multiple protein-DNA complexes with synthetic oligonucleotides containing various enhancer-binding sequences in gel mobility shift assays. The formation and stability of enzyme-DNA complexes correlate positively with kinase activation. For example, a sequence from the human collagenase AP1 enhancer binds and also activates the enzyme much better than DNA from NFκB enhancers, although even the tighter complex can be competed with sufficiently high concentrations of non-specific DNA. On the other hand, an AP1-binding enhancer sequence from the human C-*jun* gene forms a complex with purified enzyme which can not be competed by heterologous DNA. These results suggest that DNA-PK may be preferentially activated by specific DNA sequences *in vivo*. We have therefore attempted to systematically determine the DNA sequence requirements for enzyme binding by cloning oligonucleotides that form stable complexes with the enzyme. Synthetic oligonucleotides containing a continuous sequence of 16 random bases were bound to the purified kinase and then immunoprecipitated with a mixture of monoclonal antibodies against DNA-PK. DNA in the washed immunoprecipitate was amplified by PCR and re-selected by binding and immunoprecipitation. After six cycles of selection and amplification, a DNA preparation was obtained which bound specifically to the purified enzyme as judged by gel shift competition assay. Inserts from individual clones derived from the selected DNA populations are currently being re-screened by gel shift competition assays prior to sequencing.

BZ 543 REGULATED TRANSCRIPTIONAL ACTIVATION BY ELK-1 PROTEIN. Richard Marais, Stephen Dalton, Caroline Hill, Judy Wynne and Richard Treisman. Transcription Laboratory, ICRF, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, U.K.

Many genes whose transcription is transiently induced when cells are stimulated by growth factors are regulated by Serun Response Elements (SREs), which form binding sites for the transcription factor SRF. At the c-fos SRE, SRF forms a ternary complex with an accessory protein, Ternary Complex Factor, which cannot bind the SRE by itself. Two Ets-domain proteins, Elk-1 and SAP-1, display TCF activity. We have studied the role of phosphorylation in the regulation of transcriptional activation properties of Elk-1 using both transient transfection techniques and recombinant Elk-1 produced in insect cells. Following stimulation of cells with either serum or a variety of growth factors, an Elk-1 kinase activity is rapidly induced, and Elk-1 protein is phosphorylated. Concomitantly, a conformation change in the Elk-1/SRF ternary complex occurs which can be mimicked in vitro by phosphorylation of Elk-1 with MAP kinase. We have mapped MAP kinase phosphorylation sites in Elk-1, and will present results demonstrating that these sites are required for growth factor- regulated transcriptional activation by Elk-1.

BZ 544 **NF-IL6 phosphorylation by MAP kinase**

[1]Toshihiro Nakajima, [1]Shigemi Kinoshita, [2]Tatsuru Sasagawa, [2]Masanobu Naruto, [1]Shizuo Akira and [1]Tadamitsu Kishimoto. [1]Inst. Mol. Cell. Biol., Osaka Univ. [2]Basic Research Inst., Toray Ind. Inc., Japan.

NF-IL6 is a member of the bZIP family transcription factors and is involved in transcriptional regulation of many inducible genes, including IL-6, *c-fos* and acute phase proteins. NF-IL6 is also known as LAP, IL-6DBP, AGP/EBP, rNF-IL6 and C/EBPβ. We performed tryptic peptide mapping of metabolically ^{32}P-labelled NF-IL6 protein and identified Ser-231 and Thr-235 as phosphorylation sites. Comparison of amino acid sequence of each phosphorylation site with consensus sequence recognized by various protein kinases revealed that Thr-235 is a possible phosphorylation site for MAP kinase. Therefore, we prepared a synthetic peptide and performed *in vitro* kination assay. *In vitro* kination assay indicated that this peptide was phosphorylated by MAP kinases *in vitro*. Phosphoamino acid analysis of the phosphorylated peptide showed that phosphorylation occured only at threonine residue and mass spectrometry analysis revealed that phosphorylation by MAP kinases occured at Thr-235. Transient luciferase assay indicated that Thr-235 was essential for the activation of NF-IL6. These results suggest that NF-IL6 is activated by MAP kinases via phosphorylation of Thr-235.

BZ 545 TRANSCRIPTIONAL REGULATION OF c-*fos*: SRF AND ELK-1 AS TARGETS FOR PROLIFERATIVE SIGNALS, Alfred Nordheim, Raymund Zinck, Vera Pingoud, Ralf Janknecht, and Robert A. Hipskind, Institute for Molecular Biology, Hannover Medical School, Hannover, Germany

The cellular response to proliferative signals involves an instantaneous transcriptional activation of the c-*fos* proto-oncogene. The serum response element (SRE) of the c-*fos* promoter mediates this response and functions in a complex with at least two proteins, namely the serum response factor (SRF) and the ternary complex factor p62^{TCF}. SRF is a nuclear transcription factor modified by glycosylation and phosphorylation. We will provide evidence that p62^{TCF} is indistinguishable from the Ets protein Elk-1, while distinctly different to the functionally related factors SAP-1a and SAP-1b.

Additionally, we will present data arguing for Elk-1 being directly targeted by signal-activated protein modification enzymes.

BZ 546 REGULATION OF HUMAN SKELETAL MUSCLE PP-1 GENE EXPRESSION BY INSULIN, Norman, SA, D.Bruce Thompson and David Mott, National Institutes of Health, Phoenix, AZ 85016.

We have previously demonstrated that human subjects with low rates of insulin-mediated glucose disposal in vivo (insulin resistance), have reduced insulin stimulated glycogen synthase activity in skeletal muscle (1). In addition, glycogen synthase phosphatase (PP-1) activity is reduced both before and after insulin infusion in insulin resistant subjects (2). The activity differences observed are not due to differences in PP-1 protein abundance (3).

To determine whether regulation of the observed differences in PP-1 activity occurs at the level of PP-1 gene expression, we have isolated PP-1 cDNA clones from human skeletal muscle libraries. Two PP-1 cDNA clones were isolated, one being homologous to a PP-1α cDNA clone isolated from human liver (4) and the other is homologous to a PP-1γ cDNA present in rat liver (5). PP-1γ has been localized to human chromosome 12. Northern blot analysis shows PP-1α and PP-1γ mRNAs are expressed in a variety of human tissues (lung, liver, brain, heart, kidney and placenta). RNA expression of both PP-1α and PP-1γ is negatively regulated by insulin. Isolation of PP-1 genomic clones and characterization of cis-acting sequences involved in insulin response are in progress.

1. Bogardus, C. et al. (1984) J. Clin. Invest. 73: 1185-1190.
2. Kida, Y. et al. (1990) J. Clin. Invest. 85: 476-481.
3. Nyomba, B.L. et al. (1991) J. Clin. Invest. 88: 1540-1545.
4. Barker, H.M. et al. (1990) Genomics 7: 159-166.
5. Sasaki, K. et al. (1990) Jpn. J. Cancer Res. 81:1272-1280.

BZ 547 THE CHORIONIC GONADOTROPIN β5 GENE IS STIMULATED BY cAMP AND E1A THROUGH DISTINCT REGIONS, Richard G. Pestell, Noreen Troccoli, Chris Albanese, Anthony N. Hollenberg and J. Larry Jameson, Thyroid Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Chorionic gonadotropin (CG) β subunit genes are expressed in the placenta at a time of rapid cellular proliferation in a process that can be mimicked by cAMP treatment. It is also produced ectopically by many different neoplasms. The CGβ5 gene promoter has several features in common with "housekeeping" genes including the presence of multiple GC rich regions and the absence of a TATA box.

Transient expression studies performed in JEG-3 choriocarcinoma cells using 5' flanking sequences of the CGβ5 gene linked to the luciferase reporter gene identified a region between -311 and -210 that conveys basal level expression and a 9 fold induction by cAMP. This region does not contain sequences resembling a classical CRE but includes 2 regions, γ and δ, that are protected in DNAse 1 footprinting using JEG-3 nuclear extracts. Mutations within either region reduce basal level expression and cAMP responsiveness. Band-shift assays using the δ region identified several bands which were competed by the δ region probe and a single band competed by the γ region. Southwestern (SW) blot analysis of nuclear extracts from JEG-3 cells and several other cell lines using the δ region probe identified a predominant 75 kDa protein which was reduced in size with phosphatase treatment. The δ region probe did not bind E. coli expressed CREB or other proteins detected by a labelled CRE. Screening of a human placental λ GT11 library using the δ probe has identified a candidate DNA binding protein.

The ability of E1A to stimulate transcription of several "housekeeping" genes as well as genes that are induced by cAMP prompted us to determine whether E1A modulation of cAMP responsiveness was a more general property of E1A. Expression of E1A caused a 9 fold induction of -3700 CGβ5LUC and induced transcription of the CGβ5 5' flanking LUC reporter constructs. Mutations that reduced basal and cAMP induced activity did not reduce induction by E1A. These results indicate that E1A and cAMP stimulation of the CGβ5 gene are mediated through distinct regulatory mechanisms.

BZ 548 **Hepatocyte Nuclear Factor -3 (HNF-3) Transcriptional Activity is Regulated by Phosphorylation.** X. Qian and R.H. Costa, University of Illinois College of Medicine, 1853 W. Polk St., Chicago, IL. 60612.

HNF-3 is a liver transcription factor involved in the coordinate regulation of hepatocyte-specific gene expression. Three distinct HNF-3 proteins (α, β, γ) are expressed in the liver. These proteins share 95% homology in their DNA binding domains and possess conserved sequences within their transcriptional activation domains. The HNF-3 DNA binding domain defines a new gene family that includes several developmental regulatory proteins and tissue-specific transcription factors. We have demonstrated that two of the HNF-3 proteins (α, β) are phosphorylated in human HepG2 cells and that phosphorylation potentiates HNF-3 transcriptional activation. Treatment of HepG2 cells with several protein kinase activators (eg. TPA) and phosphatase inhibitors enhances the stimulation of an HNF-3 responsive reporter gene. Moreover, bacterial fusion proteins containing the HNF-3α and HNF-3β DNA binding or activating domains are differentially phosphorylated *in vitro* by a number of distinct protein kinases. This suggests that the HNF-3 proteins may be regulated by protein phosphorylation in response to different intracellular and/or extracellular signals. In addition, phosphorylation of the HNF-3α DNA binding fusion protein by protein kinase A results in enhanced DNA binding affinity, providing a functional role for one of these phosphorylation sites. Further analysis of HNF-3 mutants will provide information regarding the contribution of these phosphorylation events to the activity of the HNF-3 proteins.

BZ 549 REPRESSION OF CARDIAC GENE EXPRESSION BY A CONSTITUTIVELY ACTIVATED CA^{2+}/CALMODULIN DEPENDENT PROTEIN KINASE II, Martha T. Ramirez, Howard Schulman*, and Joan Heller Brown, Departments of Pharmacology, Stanford University* and University of California, San Diego, La Jolla, CA 92093-0636

Activation of α_1-adrenergic or endothelin receptors in neonatal rat ventricular myocytes increase $InsP_3$, diacylglycerol, and protein kinase C (PKC) activity. Long-term treatment with these agents leads to an increase in cell size and induces expression of the atrial natriuretic factor (ANF), myosin light chain-2 (MLC-2), and other cardiac specific genes. Previous experiments using phorbol esters and expression of constitutively activated forms of PKC have demonstrated that PKC mediates agonist induced change in gene expression. Other studies using pharmacological inhibitors suggest that Ca^{2+}/calmodulin dependent protein kinase II (CaM-KII) may also be a mediator of the hypertrophic changes induced by phenylephrine or endothelin. To test this hypothesis, neonatal ventricular myocytes were transfected with expression vectors containing the α subunit of CaM-KII rendered constitutively active by mutation of threonine 286 to aspartate (CaM-KII T^{286D}). Surprisingly, and in contrast to our findings with constitutively activated PKC, CaM-KII T^{286D} repressed expression of cotransfected ANF and MLC-2/luciferase reporter genes. Expression of the ANF and MLC-2 genes was decreased by about 65% in cells transfected with 0.1 ug to 10 ug of CaM-KII T^{286D}. A CaM-KII T^{286D} gene containing a second mutation which abolished catalytic activity did not inhibit ANF gene expression, indicating that repression by CaM-KII T^{286D} was dependent on its kinase activity. In addition CaM-KII T^{286D} increased (about 3-fold) luciferase activity driven by a RSV and by a recently reported CaM kinase dependent response element (CaMRE), suggesting that repression by CaM-KII T^{286D} is selective for the cardiac genes. To localize potential sites in the ANF promoter responsible for CaM-KII T^{286D} repression, we tested a series of truncation mutants. Regions that were necessary for maximal repression were localized to positions -388 to -288 and -188 to -120. Regulatory elements common to these regions are currently being identified and investigated.

BZ 550 **CELL-FREE ACTIVATION OF A DNA-BINDING PROTEIN BY EPIDERMAL GROWTH FACTOR.** Henry B. Sadowski and Michael Gilman, Cold Spring Harbor Laboratory, Box 100, Cold Spring Harbor, N.Y. 11724.

Growth factors such as PDGF and EGF bind to and activate cell surface receptors with intrinsic tyrosine kinase activities, eliciting multiple physiological changes in target cells, including alterations in cellular gene expression. Receptor tyrosine kinase signaling involves recruitment of specific proteins into a "signaling complex" through interactions between receptor autophosphorylation sites and the SH2 domains of signaling proteins. Diverse signals can subsequently be generated, depending on the specific receptor and cell type. How such signals are transmitted to the nucleus remains poorly understood, but because induction of gene transcription by growth factors occurs in the absence of new protein synthesis, one or more signals must directly affect transcription factors. Transcription of the c-*fos* gene in cells is rapidly activated by PDGF and EGF through PKC-dependent and -independent pathways. Maximal activation requires the serum response element (SRE), located 300 bp upstream of the start site. But an additional element, ~25 bp 5' of the SRE, has been implicated in c-*fos* induction by PDGF. This element (SIE) binds a protein(s), termed SIF (c-*sis* inducible factor), whose latent DNA-binding activity is activated within min after treatment of cells with PDGF. We have shown that SIF DNA-binding can be rapidly activated by several other receptor tyrosine kinases, including the EGF receptor, the insulin receptor, and the product of the *neu* oncogene. To study the mechanism by which receptor tyrosine kinases activate SIF DNA-binding, we developed a cell-free system using protein fractions from unstimulated cells. In this assay, activation of latent SIF DNA-binding requires EGF, hydrolyzable ATP, Mg and/or Mn, detergent-treated membranes, and cytoplasmic or nuclear fractions. Activation is time-, temperature-, and protein concentration-dependent, and requires the EGF-receptor. Anti-phosphotyrosine antibody blocks SIF activation *in vitro*, but does not affect SIF DNA-binding activity when added after the activation reaction, suggesting that SIF itself may not be phosphorylated on tyrosine. Nevertheless, phosphorylation of SIF is required for DNA binding, because *in vivo*-activated SIF is inactivated by phosphatase treatment. Together these data suggest that SIF may be activated by a protein kinase cascade that is active in our *in vitro* reaction. Finally, addition of an *E. coli*-produced SH2 domain to the reaction inhibits SIF activation in a dose-dependent fashion, implicating SH2 domains in the activation process.

BZ 551 **DEPHOSPHORYLATION OF A NUCLEAR ACTIN BINDING PROTEIN: AN ACCESSORY RECEPTOR DEPENDENT SIGNALLING EVENT IN T LYMPHOCYTES** Yvonne Samstag, Christoph Eckerskorn, Doris Schiller, Stefan W. Henning, Friedrich Lottspeich and Stefan C. Meuer, Applied Immunology, German Cancer Research Center, Heidelberg, FRG

In order to identify signalling events specifically induced through accessory molecules (i.e. CD2), we have compared protein phosphorylation events events in human T lymphocytes following triggering of the antigen receptor (TCR/CD3) or, alternatively, the accessory molecule CD2. By means of 2D-gel electrophoresis we have recently identified a 19 kDa cytosolic phosphoprotein (pp19) with several serine phosphorylation sites. At least one of these sites is transiently dephosphorylated by an as yet unknown serine phosphatase following triggering through CD2 but not TCR/CD3. With similar kinetics, the partially dephosphorylated form of pp19 translocates to the nuclear compartment suggesting that pp19 is involved in signal transmission from the cell surface into the nucleus. Dephosphorylation of pp19 correlates with functional responses involved in the induction of T cell proliferation, *namely* responsiveness to IL-6 and the production of IL-2. In contrast, IFN γ synthesis does not show any relationship to pp19 dephosphorylation. Interestingly, when we analyzed the autonomously growing T lymphoma line Jurkat, spontaneous dephosphorylation of pp19, independent of an extracellular signal, was observed. This implies that pp19 may be involved in malignant T cell growth. We have purified pp19 for protein sequencing. The aminoacid sequence of four peptides has been obtained and reveals homology to an 19kDa actin binding protein. This protein contains a region homologous to the nuclear translocation signal sequence of SV40 large T antigen. Moreover, in fibroblasts it is known to translocate into the nucleus following exposure of the cells to heat shock. These data suggest that serine phosphatases as well as actin binding proteins are important molecules involved in signal transduction during lymphocyte development and activation.

BZ 552 TRANSFORMATION BY THE BCR-ABL GENE OF CHRONIC MYELOGENOUS LEUKEMIA: ROLES FOR MYC AND MAX. Charles L. Sawyers, Wendy A. Callahan and Owen N. Witte. Departments of Medicine, Hematology-Oncology, and Microbiology and Molecular Genetics, Howard Hughes Medical Institute, University of California - Los Angeles, Los Angeles, CA 90024.

C-abl is a tyrosine kinase proto-oncogene localized in the nucleus with cell cycle specific DNA binding activity. The naturally occurring leukemogenic forms, v-abl and BCR-ABL, localize to the cytoplasm and do not bind DNA. We have used dominant negative mutants of potential downstream molecules to understand the signal transduction pathways of the ABL proteins. By overexpressing dominant negative mutations in c-myc, we have recently demonstrated that transformation of fibroblasts and of bone marrow cells by both v-abl and BCR-ABL requires myc, suggesting that the BCR-ABL transformation signal may be mediated, in part, through myc. We have now examined the role of the related genes N-myc and max. In a rat-1 fibroblast transformation model, overexpression of N-myc enhanced soft agar colony formation by both BCR-ABL and v-abl by more than 200 percent, to levels similar to those observed with overexpression of c-myc. This finding suggests functional homology between N-myc and c-myc for BCR-ABL transformation. In contrast, overexpression of max, a DNA binding partner for either c-myc or N-myc, suppressed transformation by both BCR-ABL and v-ABL by 80-90 percent. Co-expression of myc and max in the same cell rescued the suppressed phenotype, suggesting that the relative amounts of myc and max in the cell determine the effect on transformation. This model suggests that max-max homodimers have a suppressive effect on BCR-ABL transformation, whereas myc-max heterodimers have a stimulatory effect.

BZ 553 OPPOSING EFFECTS OF KINASE AND PHOSPHATASE ACTIVITIES ON THE PHOSPHORYLATION STATE OF GROWTH SUPPRESSOR P53 - CORRELATION WITH CELLULAR TRANSFORMATION.

Karl Heinz Scheidtmann, Elke Müller and Gerd Landsberg; University of Bonn, Institute of Genetics, D-5300 Bonn 1, FR Germany.

The cellular protein p53 plays an important role in growth control. Its activity may be regulated by phosphorylation, in analogy to the Rb protein. Phosphorylation of p53 is enhanced upon infection or transformation of cells with wild-type (wt) SV40 but not with transformation-defective (td) mutants (1). Moreover, in revertant cells, p53 assumes its normal phosphorylation state. Thus, there is a tight correlation between the phosphorylation state of p53 and the transformed state of the cell. Enhanced phosphorylation may result from upregulation of protein kinases or downregulation of phosphatases. To investigate these possibilities in more detail, phosphorylation of p53 was studied in vivo and in vitro, employing kinase or phosphatase inhibitors in vivo or kinase or phosphatase reactions in vitro, followed by two-dimensional phosphopeptide mapping. The wt and td-transformants clearly differed in kinase activity, whereas phosphatase activity was comparable. In contrast, the revertant cells showed enhanced phosphatase activity compared to the parental transformed cells, whereas kinase activity was comparable. The data indicate that transformation by SV40 T antigen is mediated, in part, by activation of kinases leading to enhanced phosphorylation of p53, whereas reversion to the normal phenotype may be achieved by activation of counteracting phosphatases. Experiments are in progress to modulate phosphatase activity and thereby the transformation state of the cells.

1) Scheidtmann & Haber, J. Virol. 64, 672, 1990

Supported by Deutsche Forschungsgemeinschaft, grant Sche246/4-1

BZ 554 LY-GDI, A LYMPHOID-SPECIFIC GDP/GTP EXCHANGE PROTEIN FOR P21^{rho}, Peggy Scherle, Timothy Behrens and Louis Staudt, Metabolism Branch, National Cancer Institute, Bethesda, MD 20892

The ras-related, small GTP-binding proteins are active only in the GTP-bound state and are involved in cellular events such as cell signalling, proliferation, cytoskeletal structure and trafficking. The activation state of the GTP-binding proteins is directly regulated by at least three proteins: a GTP-ase activating protein, GAP, which stimulates the intrinsic GTP-ase activity of the GTP-binding protein; guanine-nucleotide-releasing factors which stimulate GDP/GTP exchange; and exchange inhibitors. The regulatory proteins are, in general, ubiquitously expressed. Changes in their relative activites is one way the activation state of the GTP-binding protein is regulated in response to external stimuli. The existence of cell-type specific regulatory proteins may offer another means of regulating GTP-binding proteins. By utilizing a subtractive hybridization approach, we have isolated 12 distinct cDNA sequences which correspond to genes expressed in human B and T cell lines but not in the erythroleukia cell line, K562. One of these genes has > 80% homology to a previously cloned bovine gene shown to be a GDP/GTP exchange inhibitor (GDI) for p21^{rho}. In contrast to the bovine GDI, which is ubiquitously expressed, the gene that we have isolated is expressed only in hematopoietic tissues and in B and T cell lines. We refer to this gene as lymphoid-restricted GDI (Ly-GDI). The full-length cDNA encodes a 27 kd protein. By immunofluorescence staining with an antibody specific for Ly-GDI, we demonstrated that the protein is localized predominantly in the cytoplasm. Binding studies both in vitro and in vivo demonstrate that Ly-GDI interacts specifically with p21^{rhoA}. Using affinity chromatography with a bacterially expressed Ly-GDI fusion protein, we can isolate rhoA, but not ras or rap, from cell lysates. In addition, immunoprecipitation of Ly-GDI coprecipitates rhoA. A region of approximately 70 amino acids at the N-terminus of Ly-GDI is important for binding to rhoA and is weakly homologous to ras-GAP. Our results point to a role of Ly-GDI and rhoA in lymphocyte-specific signalling events.

BZ 555 PHOSPHORYLATION OF HTLV TAX IS NECESSARY FOR EFFICIENT TRANSACTIVATION OF THE PROVIRAL LTR, Ping Shen, Ian B. Murton, Tracy L. Martin, and William Wachsman, Research Service, San Diego VAMC and Division of Hematology/Oncology, UCSD School of Medicine, La Jolla, CA 92093-0677

The human T-lymphotropic viruses (HTLV) types 1 and 2 are the etiologic agents of specific types of T-cell leukemia and lymphoma. HTLV also acutely transforms T-cells in vitro. Tax, a HTLV-encoded accessory gene product is required for efficient viral replication and causes aberrant expression of several T-cell genes involved in cell growth and proliferation. Thus, it is thought to serve a function in the process of HTLV-induced malignancy. The basis for these actions of Tax is poorly understood and are thought to be mediated, indirectly, through cellular regulatory factors. Tax is known to be Ser/Thr phosphorylated, however, the role that this post-translational modification plays with respect to Tax function is unknown.

We have performed 2-D phosphopeptide mapping of trypsin digested HTLV-1 Tax (Tax1). Three phosphopeptides were detected, indicating that Tax1 is phosphorylated at multiple sites. Analysis of Tax1 and Tax2 sequences from 5 HTLV isolates revealed 40 conserved Ser/Thr residues. Using site-directed mutagenesis we substituted Ala for Ser/Thr at 9 of the conserved residues in Tax2. The function of these mutants was compared to wild-type *tax2* by transient cotransfection with a HTLV-1 LTR/CAT reporter construct into the Jurkat human T-cell line. Four of the 9 mutants functioned similarly to wild-type *tax2*, while 4 others exhibited a 2 to 5-fold reduction in activity on the HTLV-1 LTR. In contrast, one mutant, Tax2$_{113T\text{-}A}$, was found to be virtually inactive. Similarly, mutants of *tax1*, in which Ala, Asp, or Gln were substituted for this conserved Ser residue 113 were also markedly reduced in activity on the HTLV-1 LTR in comparison to wild-type *tax1*. Reversal of the substitution to Ser in Tax1 restored activity. However, when this residue was changed to Thr, transactivation of the HTLV-1 LTR was increased 20-fold with respect to wild-type Tax1. Sequences surrounding this conserved Ser/Thr residue in Tax1 and Tax2 suggest that it is a potential target consensus site for phosphorylation by PKC, PKA, and/or p34cdc2 kinase (RKYSPFR/Tax1; RKHTPYR/Tax2). These results suggest that phosphorylation of this conserved Ser/Thr residue 113 in Tax is necessary for efficient transactivation of the HTLV-1 LTR in human T cells. We are now determining which, if any or all, of the 3 potential signal transduction pathways are involved in HTLV Tax phosphorylation.

BZ 556 EXPRESSION OF BCR-ABL INDUCES TRANSCRIPTION OF GROWTH FACTOR GENES IN A HUMAN FACTOR-DEPENDENT HEMATOPOIETIC M07E CELLS AND ALTERS THE DIFFERENTIATION AND PROLIFERATION PROGRAM. C. Sirard, P. Laneuville*, T. Lapidot, J. E. Dick. Dept. of Genetics, Research Institute, Hospital for Sick Children; and Dept. of Molecular and Medical Genetics, University of Toronto; *Division of Hematology and Medical Oncology, Royal Victoria Hospital, McGill University, Montreal, Quebec.

The introduction of a retrovirus vector expressing p210$^{bcr\text{-}abl}$ into the human factor-dependent cell line M07E results in the rapid outgrowth of factor-independent (FI) cells that remain responsive to exogenous growth factors. RT-PCR analysis showed that the GM-CSF and IL-3 growth factor genes were transcriptionally activated in clonal populations, early after *bcr-abl* infection. Concentrated conditioned media (CM) from the factor-independent cells contained mostly biologically active GM-CSF and to a lesser extent IL-3, suggesting that the growth autonomy of the *bcr-abl*-expressing M07E cells was acquired via an autocrine mechanism. Neutralizing antibodies to these growth factors partially inhibited cell growth implying that the newly produced growth factors may also bind to their cognate receptors internally. Experiments using anti-sense oligos directed against the growth factors are currently in progress. In addition to factor-independent growth, *bcr-abl*-expressing M07E cells acquired a more mature megakaryocytic phenotype compared to control M07E cells. The M07E-differentiating activity was detected only in the presence of lethally irradiated factor-independent cells and not with its concentrated CM, suggesting a cell membrane associated activity. Furthermore, expression of *bcr-abl* also considerably reduced the generation time of M07E cells *in vitro*. We have shown that the MO7E cells and other myeloid cells proliferate preferentially to the marrow of immune-deficient scid mice. The proliferative advantage of the FI cells also took place *in vivo* using a competition assay where mixtures of parental and FI populations were simutaneously injected in these recepients. These data provide experimental evidence that expression of *bcr-abl* tyrosine kinase in human hematopoietic cells activated transcription of growth factor genes resulting in a pleiotropic effect on growth factor requirements, proliferation and differentiation.

BZ 557 ACTIVITY OF THE DNA-DEPENDENT PROTEIN KINASE (DNA-PK) MAY REQUIRE TWO DISTINCT PROTEINS. Shi-shin Sun, Nusrat Parveen Malik, Elissa Lugo-Ayala, Ivana Vancurova, and Timothy H. Carter, Dept. of Biological Sciences, St. John's University, Jamaica, NY 11439

A protein kinase which requires the presence of double-stranded DNA (DNA-PK) has been purified in several laboratories (Carter et al., Mol. Cell Biol. *10*, 6460, 1990; Lees-Miller et al., Mol. Cell Biol. *10*, 6472, 1990) and identified as a 350 kDa polypeptide. This conclusion was based primarily on the ability of a monoclonal antibody (mab) against the 350 kDa polypeptide, 18-2, to inhibit enzyme activity and to retard the mobility of a large DNA-protein complex formed by the purified enzyme. However, we have found that a second mab, 8-2, prevents the formation of all DNA-protein complexes by purified enzyme in a gel shift assay, including a high mobility complex that does not contain the 350 kDa polypeptide. These data suggest that the 350 kDa protein binds DNA as a ternary complex with a smaller DNA-binding protein. Curiously, mab8-2 can also activate the purified kinase in the absence of DNA. This mab does not recognize the 350 kDa polypeptide on Western blots, and immunoprecipitates predominantly a 176 kDa polypeptide from ^{35}S-labeled whole cell extracts. The immunoprecipitates contain substantial protein kinase activity in the absence of added DNA and only small amounts of the 350 kDa species. Because these experiments were done with crude extracts, however, it is not yet possible to say whether the kinase activity immunoprecipitated by mab8-2 reflects activated DNA-PK or another co-precipitating (DNA-independent) enzyme. In contrast, another mab against the 350 kDa polypeptide, 25-4, immunoprecipitates primarily the 350 kDa species from crude extracts, but this immunoprecipitate has relatively little kinase activity. Taken together, these results suggest that the phosphotransferase activity and the DNA-dependence of DNA-PK require at least one protein in addition to the 350kDa species.

BZ 558 DNA-ACTIVATED PROTEIN KINASE IN RAJI BURKITT'S LYMPHOMA CELLS: PHOSPHORYLATION OF NUCLEAR PROTEIN FACTORS INVOLVED IN DNA REPLICATION AND TRANSCRIPTION. Hirobumi Teraoka, Fumiaki Watanabe, Hanae Minami, Shigeyuki Iijima, Kinji Tsukada, Takayasu Date* and Shigetaka Kitajima, Medical Research Institute, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101, Japan and *Kanazawa Medical University, Kahoku-gun, Ishikawa 920-02, Japan

We recently reported that c-Myc proto-oncoprotein is phosphorylated in vitro by relaxed, double-stranded DNA-activated protein kinase (DNA-PK) from Raji Burkitt's lymphoma cells (Eur. J. Biochem. 206, 595, '92). In addition to c-Myc, phosphorylation of some other nuclear protein factors involved in DNA replication and transcription by DNA-PK has been investigated. DNA ligase I that is responsible for DNA replication process was purified from E. coli cells harboring an expressible cDNA construct for full-length human DNA ligase I. The recombinant DNA ligase I expressed full activity and treatment of the enzyme with alkaline phosphatase resulted in decrease in the activity. The DNA ligase I was susceptible to phosphorylation by DNA-PK as well as by casein kinase II and cdc2 kinase without any significant effects on the ligation activity. DNA polymerase β involved in DNA repair and recombination was not phosphorylated by DNA-PK. Human TFIID, one of general transcription factors for RNA polymerase II, was phosphorylated by DNA-PK in a DNA-dependent manner. In the in vitro phosphorylation of TFIID by DNA-PK, at least 0.5 mol phosphate per mol protein was incorporated into serine residues. In contrast, phosphorylation of TFIIF by DNA-PK was negligible. Studies on the effect of TFIID-phosphorylation on in vitro transcription activity are in progress.

BZ 559 THE "PRIMING" PHOSPHORYLATION OF pRB IN G_{1A} IS INHIBITED DURING CELL CYCLE ARREST BY α-IFN. Shaun Thomas and Alison Bybee, Department of Haematology, University College and Middlesex School of Medicine, 98 Chenies Mews, London WC1E 6HX, England.

The retinoblastoma protein, pRB, can be multiply phosphorylated from a basal, hypo-phosphorylated form. The degree of phosphorylation is cell cycle dependent and phosphorylation changes are thought to control cell cycle progression. Actively cycling human cells contain partially phosphorylated pRB in early G_1 (G_{1A}) (Burke *et al.*1992 *Oncogene* **7**: 783) and these intermediate forms of pRB become further phosphorylated to a hyper-phosphorylated form as cells pass into late G_1 (G_{1B}). In contrast, cell cycle arrest of a human B-cell line (Daudi) by α-IFN results in an accumulation of cells in G_1 and these cells contain the hypo-phosphorylated form of pRB in both G_{1A} and G_{1B} (*ibid.*; Thomas *et al.* 1991 *Oncogene* **6**: 317). Hypo-phosphorylated pRB is detectable in G_1 within 2-4h of α-IFN addition, but pRB is not dephosphorylated in other cell cycle phases even though other events stimulated by α-IFN, such as a decrease in *c-myc* protein levels or induction of the DAI kinase, are not restricted to G_1. Since hypo-phosphorylated pRB is thought to prevent cell proliferation, our data would suggest that cell cycle arrest induced by α-IFN may be mediated by preventing the initial phosphorylation of pRB during or prior to G_{1A}. In order to prevent pRB being phosphorylated, α-IFN must inhibit kinase activity and/or induce phosphatase activity against pRB. α-IFN does not increase phosphate turnover on pRB, therefore the decrease in pRB phosphorylation is probably due to decreased kinase activity. One candidate pRB kinase is $p34^{cdc2}$/$p58^{cyclinA}$. The phosphorylation of $p34^{cdc2}$ (Thomas 1989 *J. Biol. Chem.* **264**: 13697) and its kinase activity against a pRB peptide is inhibited by α-IFN. Further, α-IFN inhibits $p58^{cyclinA}$ synthesis. In cycling cells $p58^{cyclinA}$ synthesis occurs during G_{1B}/early S phase, coincident with the phosphorylation of $p34^{cdc2}$ (Bybee and Thomas 1992 *Biochem. Biophys. Acta*: in press). Thus, $p34^{cdc2}$/$p58^{cyclinA}$ probably does not cause the initial, partial phosphorylation of pRB in G_{1A}, and so at least one other unidentified kinase, also α-IFN sensitive, is needed to "prime" the phosphorylation of pRB in G_{1A}. The phosphorylation and kinase activity of $p33^{cdk2}$ is also inhibited by α-IFN but it is not yet clear whether $p33^{cdk2}$ is involved in phosphorylating pRB in G_{1A}. The identity of the pRB "priming" kinase(s) is currently under investigation. Supported by the C. R. C. and Kay Kendall Leukaemia Fund.

BZ 560 STUDY OF A CTD KINASE ACTIVITY INCREASED BY HEAT-CHOCK TREATMENT, Sylviane TRIGON, Liliana PASLARU and Michel MORANGE. Ecole Normale Supérieure, 46 rue d'Ulm, 75230 PARIS Cedex 05 France

Cellular Stresses induce a decrease in transcriptional activity and protein synthesis concomitant with an increase of heat-shock protein gene expression. These events are preceded by rapid modifications, such as an alteration in the pattern of phosphorylated proteins, which do not require protein synthesis. It has been previously shown that a CTD kinase activity is increased after heat-shock treatment (HS-CTD kinase). Eukaryotic RNA polymerase II largest subunit contains a C-terminal domain (CTD) formed of SPTSPSY contiguous repeated motifs. HS-CTD kinase activity is detected by *in vitro* phosphorylation of a synthetic tetramer of the heptapeptide SPTSPSY. HS-CTD kinase purification is currently in progress. The activity is bound on a DEAE-cellulose resin at pH 7.4 and can be eluted by a glycerophosphate gradient. It is also bound by two reactive dyes: cibacron blue and red-120 agarose and an hydrophobic one: phenyl sepharose.

Only the serines present in the repeated SPTSPSY motif are phosphorylated by the HS-CTD kinase activity. To study which of the three serines are phosphorylated, we have synthesized different peptides, where serines have been successively replaced by alanines, containing one or two SPTSPSY motifs. Using these different peptides, we have shown that only the central serine of the motif is phosphorylated. This serine is next to a proline residue, the role of which remains to be determined.

In order to characterize the requirements of the HS-CTD kinase for the aminoacids surrounding the phosphorylated serine residue, we are presently measuring the activity of the HS-CTD kinase on different peptides, either neo-synthesized or isolated from proteins containing repetitive sequences similar to the RNA polymerase CTD-domain.

BZ 561 CONTROL OF SV40 DNA REPLICATION BY THE RECIPROCAL ACTION OF CASEIN KINASE I AND PROTEIN PHOSPHATASE $2A_c$ David M. Virshup, A. Cegielska, K. Fish, S. Shaffer, D. Hillam, Program in Human Molecular Biology and Genetics, University of Utah, Salt Lake City, UT 84112

The study of simian virus 40 minichromosome replication has produced many insights into the control and enzymology of mammalian DNA replication. The initiation of SV40 DNA replication is regulated by the phosphorylation state of the viral initiator protein, large T antigen. Unwinding of the origin of replication is markedly stimulated by the specific dephosphorylation of T antigen by the cellular protein phosphatase $2A_c$. Dephosphorylation of T antigen permits specific protein-protein interactions between the two T antigen hexamers bound to the origin of replication. These interactions are required for the double hexamer to melt the origin region, thus generating a single stranded template for new strand synthesis. Addition of protein phosphatase $2A_c$ to replication extracts from G1 phase cells stimulates *in vitro* SV40 DNA replication over 20-fold, up to the level of replication seen in S phase cell extracts.

Using a functional assay, we have purified from HeLa cell nuclear extracts a 35 kDa serine/threonine protein kinase. This kinase phosphorylates T antigen at sites that are phosphorylated *in vivo* and inhibits its ability to initiate SV40 DNA replication *in vitro*. The inhibition of both origin unwinding and DNA replication by the kinase is reversed by protein phosphatase $2A_c$. As determined by molecular weight, substrate specificity, autophosphorylation, immunoreactivity and limited sequence analysis, this kinase appears to be a nuclear form of casein kinase I (CKI). CKI is a ubiquitous serine/threonine protein kinase that is closely related to a yeast protein kinase (HRR25) involved in DNA metabolism. The HeLa cell phosphorylation cycle that controls the initiation of SV40 DNA replication may also play a role in cellular DNA metabolism.

BZ 562 TCF1α/hLEF ACTIVATES TRANSCRIPTION THROUGH A PHOSPHORYLATED DOMAIN DISTINCT FROM ITS DNA-BINDING DOMAIN. Marian Waterman and Katherine A. Jones, The Salk Institute, La Jolla, CA 92037

TCF1α/hLEF is a T cell and pre-B cell specific transcription factor that binds to regulatory regions active in lymphocytes. One of these regions is the T cell receptor α subunit enhancer, a powerful T cell-specific enhancer located 4.5 and 3.0 kb downstream of the α subunit gene in human and mouse respectively. TCF1α/hLEF binds to the central element of three in the 100 base pair core enhancer. Its binding is essential for enhancer activity, as are the other two elements. We and others have recently cloned TCF1α/hLEF mRNA and determined it to belong to the recently described family of DNA binding proteins termed the HMG box family. HMG domains from several members of this diverse family have been shown to bind to altered DNA structures, including cruciforms, stem-loop structures, and DNA unwound and bent by the antitumour agent cisplatin. Giese et al. (Genes Dev. 5: 2567, 1991) have shown that mLEF-1 can induce a dramatic 130° bend in double stranded DNA, indicating that HMG proteins can further distort the DNA after binding. In addition to its binding characteristics, hLEF can stimulate transcription through a transactivation domain distinct from the HMG box/DNA binding domain. This transactivation domain (amino acid 80 - 256) is T cell-specific, not a member of any known class of activation domain and is transferrable to the yeast Gal 4 DNA binding domain. Removal of the first 67 amino acids unmasks the transactivating potential of this domain. TCF1α/hLEF is phosphorylated when T cells are activated with phorbol ester and lectin treatment. Phosphate is incorporated on a specific serine residue within the transactivation domain. Mutation of this residue to alanine reduces transcriptional activation, and mutation to aspartate to mimic the charge of phospho-serine restores activity. In vitro phosphorylation with $p44^{mpk}$ Map kinase and cdc2 kinase phosphorylate bacterially expressed TCF1α/hLEF on the same serine residue. Thus TCF1α/hLEF may be a substrate for these kinases *in vivo* and might participate in the second messenger pathway initiated upon antigen recognition by T cell receptors on the cell surface.

BZ 563 PHOSPHORYLATION OF THYROID TRANSCRIPTION FACTOR 1 (TTF-1) AND ITS TRANSCRIPTIONAL ACTIVITY ARE DOWN REGULATED BY THE HA-RAS ONCOGENE, Mariastella Zannini[1], Mario De Felice[1], Alfredo Fusco[2], Maria Teresa Berlingieri[2] and Roberto Di Lauro[1], [1]Stazione Zoologica A. Dohrn, Villa Comunale, 80121, Naples, Italy and [2]Dipartimento di Biologia e Patologia Cellulare e Molecolare, Via Pansini 5, 80131, Naples, Italy.

TTF-1, a homeodomain-containing transcription factor, is required for thyroid specific expression of the thyroglobulin (Tg) and thyroperoxidase (TPO) gene promoters. Co-transfection of a TTF-1 expression vector with a Tg or TPO reporter constructs into several non-thyroid cell lines results in a specific transactivaton of the Tg and TPO promoters. On the other hand, TTF-1 protein has been found in tissues other than the thyroid and in the thyroid bud 5 days before Tg and TPO mRNAs, indicating that its presence is not sufficient to activate thyroid specific transcription. An in vitro counterpart of these situations is found in thyroid cells transformed by the Ha-ras oncogene. In these cells nearly wild-type levels of TTF-1, fully active in DNA binding, are detected. This suggests that the Ha-ras oncogene interferes with transcriptional activation by TTF-1 and not with its DNA binding properties. Phosphorylation is one of the most frequent post-translational modifications of transcription factors and has been implicated in the modulation of their transcriptional activating properties in response to extracellular signals. Immunoprecipitation of ^{32}[P]-labelled thyroid cells (FRTL-5) shows that TTF-1 is phosphorylated in serine residues. The same result is observed in extracts of ^{32}[P]-labelled FRTL-5-Ha-ras cells. However the intensity of the ^{32}[P]-labelled TTF-1 band in this cell line is clearly diminished suggesting that the Ha-ras oncogene could interfere with the phosphorylation of TTF-1.

We have identified two major phosphorylated peptides within TTF-1 and we are in the process of mapping the phosphorylation sites to test whether they are relevant for the transcriptional activity of TTF-1.

BZ 564 REGULATION OF MYOGENIN TRANSCRIPTION ACTIVITY BY PHOSPHORYLATION, Jumin Zhou, Li Li and Eric N. Olson, Department of Biochemistry and molecular Biology, M.D. Anderson Cancer Center, University of Texas Health Science Center at Houston, Houston, Texas 77030

Myogenin, a nuclear phosphoprotein, belongs to a new identified MyoD family of tissue-specific transcription factors. Each of these members, when transfected into tissue culture cells, is able to convert many cell types to muscle lineage. They share a basic-helix-loop-helix motif, and when heterodimerized with E2A gene product, another family of BHLH protein, can bind to CANNTG, termed E-box, which is present in the enhancer of most muscle specific genes, and activate transcription. Myogenin's transcription activity is down-regulated by mitogen stimulation, serum, growth factors and a number of activated oncogenes. In this poster, we are going to present our work on the roles of phosphorylation in the transcription activation and regulation of myogenin.

PKC PHOSPHORYLATION OF MYOGENIN INHIBITS ITS DNA BINDING.
As has been shown by Li et al(submitted), protein kinase C (PKC) is able to phosphorylate myogenin, in vitro and in vivo and thus inhibit myogenin DNA binding. We will show here that myogenin is phosphorylated on Thr-87 by FGF induction and okadaic acid treatment. These data further demonstrated that phosphorylation of DNA binding domain which negatively regulates myogenin DNA binding, serves as a mechanism of growth factor and oncogene repression.

N-TERMINAL PHOSPHORYLATION BY SERUM INDUCIBLE KINASE NEGATIVELY REGULATES TRANSCRIPTION OF MYOGENIN.
Beside the phosphorylation on the basic domain, the N-,and C-terminal transactivation domain are also phosphorylated and regulated. Ser43, comprising proline-directed-protein kinase phosphorylation site, is phosphorylated in vivo, and is induced by serum in a heterodimerization dependent manner. Mutation of Ser43 into Ala43 increases myogenin transcription activity several folds. Interestingly CdC2 is able to phosphorylate this site. Indicating that cell cycle related protein kinases could phosphorylate this site and negatively regulate myogenin transcription activity by phosphorylating this site.

Part of the C-terminal phosphorylation site has been mapped to Ser170, phosphorylation of the C-terminal sites is inhibited by N-terminal phosphorylation, as 43 S-A mutation increase the C-term phosphorylation 10-fold.It is interesting to notice that both phosphorylation site are proline-directed-protein kinase sites, and the C-terminal site is also proceded by 4 CK II/GSK III sites. The function of these phosphorylation sites will be discussed.

BZ 565 THE NEUROTRANSMITTER **NITRIC OXIDE INDUCES *c-fos* AND *jun B*** PROTO-ONCOGENE TRANSCRIPTION. INVOLVEMENT OF PROTEIN PHOSPHORYLATION, Jean Zwiller, Christelle Haby and Dominique Aunis, INSERM U338, 67084 Strasbourg, France.

The stimulation of various second messenger pathways induces distinct patterns of immediate early gene expression. However, little is known about the early genes induced via the stimulation of the cyclic GMP pathway. We looked at the expression of early genes of the *fos* and *jun* families in pheochromocytoma PC12 and retinoblastoma Y79 cell lines after activation of cytosolic guanylate cyclase by sodium nitroprusside. This compound spontaneously releases NO, a molecule known to be involved in cell communication. We found that expression of *c-fos* and *jun B* but not of *c-jun* nor *jun D* is increased upon activation of the cyclic GMP pathway. *c-fos* mRNA expression was the most activated (4-fold at 30 min) while *jun B* response was more modest (2.2-fold activation at 60 min). This stimulation pattern appears similar to that observed when the cAMP or the Ca^{2+} pathway is activated. On gel shift assay, nuclear extracts of NO-stimulated cells showed increased binding capacity to the AP1 binding site. The enhancement in binding ability was abolished by KT5823, an inhibitor of protein kinase G. A synergistic effect was observed when cells were treated with sodium nitroprusside and the protein phosphatase inhibitor okadaic acid. These observations show that NO increases AP1 binding capacity by mechanisms involving serine/threonine protein phosphorylation.

Protein Phosphatases

BZ 600 CD45 CONTROLS PHOSPHORYLATION AND SIGNAL TRANSDUCTION OF THE HIGH AFFINITY IgE-RECEPTOR, Martin Adamczewski[1], Gary A. Koretzky[2], and Jean-Pierre Kinet[1], [1] NIH-NIAID, Rockville, MD 20852, [2] Dept. of Int. Med., College of Med., Univ. of Iowa, Iowa City, IA 52242

The high affinity receptor for IgE on mast cells, along with the antigen receptors on T and B cells and Fc receptors for IgG, belongs to a class of receptors which lack intrinsic kinase activity, but activate non-receptor tyrosine and serine/threonine kinases. Receptor engagement triggers a chain of signaling events leading from immediate phosphorylation of receptor subunits and other proteins to activation of phosphatidylinositol-specific phospholipase C, an increase in intracellular calcium levels, and ultimately the activation of more specialized functions. Our group has shown that receptor phosphorylation is a coupling mechanism linking the receptor to other members of the signal transduction chain. IgE-receptor disengagement leads to reversal of phosphorylation by undefined phosphatases and to inhibition of activation pathways. By analysing Jurkat T cells transfected with the three subunits of the IgE-receptor and their CD45-deficient mutant counterparts we show that the activation-induced phosphorylation of the receptor and all subsequent activation events require the transmembrane tyrosine phosphatase Leukocyte Common Antigen (CD45). This implicates CD45 as one of the most receptor-proximal members of the signal transduction chain of the high affinity IgE receptor.

BZ 601 **THE CD45 TYROSINE PHOSPHATASE ACTIVATES THE p59^{fyn} TYROSINE KINASE IN HPB-ALL T CELLS.** Denis Alexander*, Lindsey Goff#, Mark Biffen*, Emer Shivnan* & Masahiro Shiroo*. *Dept. of Immunology, Institute of Animal Physiology & Genetics Research, Babraham, Cambridge, CB2 4AT; #ICRF Human Tumour Immunology Group, London, U.K.

The role of the CD45 phosphotyrosine phosphatase in coupling the T cell antigen receptor complex (TCR) to intracellular signals was investigated. CD45$^-$ HPB-ALL T cells were transfected with cDNA encoding the CD45RA$^+$B$^+$C$^-$ isoform. The tyrosine kinase activity of p59^{fyn} was found to be 65% less in CD45$^-$ cells than in CD45$^+$ cells, whereas p56^{lck} kinase activity was comparable in both sub-clones. In CD45$^-$ cells the TCR was uncoupled from protein tyrosine phosphorylation, phospholipase $C_{\gamma 1}$ *regulation*, inositol phosphate production, calcium signals, diacylglycerol production and protein kinase C activation. Restoration of TCR-coupling to all these pathways correlated with the increased p59^{fyn} activity observed in CD45-transfected cells. Co-aggregation of CD4- or CD8-p56^{lck} kinase with the TCR in CD45$^-$ cells restored TCR-induced protein tyrosine phosphorylation, phospholipase $C_{\gamma 1}$ regulation and calcium signals. Receptor-mediated calcium signals were largely due to Ca^{2+} influx, and only a minor component was caused by Ca^{2+} release from intracellular stores. Maximal CD3-mediated Ca^{2+} influx occurred at CD3 mAb concentrations at which inositol phosphate production was non-detectable. These results indicate that CD45-regulated p59^{fyn} plays a critical role in coupling the TCR to specific intracellular signalling pathways and that CD4- or CD8-p56^{lck} can only restore signal transduction coupling in CD45$^-$ cells when brought into close association with the TCR.

BZ 602 A XENOPUS LEUKOCYTE SPECIFIC ANTIGEN EXPRESSED EARLY IN DEVELOPMENT POSSESS PROTEIN TYROSINE PHOSPHATASE ACTIVITY, Laura Barritt and James B. Turpen, Department of Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, NE 68198-6395

The monoclonal antibody C121 recognizes an antigenic determinant ranging from 180 to 220 kDa which is uniquely expressed on the surface of Xenopus leukocytes. Through flow cytometric analysis and immunoprecipitation it has been demonstrated that splenic IgM+ B cells express only the highest molecular weight form whereas a 180 kDa molecule is expressed on thymocytes. Previously, analysis of lateral plate mesoderm revealed that the leukocyte specific antigen is first expressed at 48 hours in development prior to morphological differentiation of hematopoietic cells. Preliminary experiments utilizing either ^{32}P-labeled [tyr(P)] Raytide or p-nitrophenyl phosphate as a substrate demonstrated that the immunoprecipitated C121 antigen has protein tyrosine phosphatase activity. Data based on molecular weight, tissue distribution, and enzymatic activity suggest that the C121 determinant may be the amphibian homologue to members of the Leukocyte Common Antigen (L-CA) family. By analogy theses results suggest that the C121 determinant may be an accessory component in signal transduction. The early expression of the C121 determinant may indicate its involvement in intial lineage specification within the hematopoietic system.

BZ 603 REGULATION OF PROTEIN SERINE/THREONINE PHOSPHATASE 1 BY INSULIN IN RAT ADIPOCYTES, Begum N and Draznin B, Diabetes research lab, Winthrop University Hospital,Mineola,N.Y 11501 and Division of Endocrinology, V.A. Medical Center Denver, CO 80220

We investigated the subcellular distribution of protein ser/thr phosphatase 1 (PP-1) in freshly isolated rat adipocytes in the basal state and after insulin treatment. In the basal state,66% and 55% of phosphatase activity(PP-1+PP-2A) existed in the spontaneous, active form in the whole cell extracts(H) and particulate fractions(PF). In contrast,in the cytosolic fractions(CF) the entire phosphatase activity was in the active form. 50-60% of PP-1 activity(assayed in the presence of 3 nM okadaic acid) existed in the active form in H and PF and 40-50% was present in the latent inactive form. Trypsin treatment (20 ug/ml x 5min) converted the 'latent'PP-1 into an active form due to destruction of inhibitors. CF PP-1 was present entirely in the active form and hence trypsin did not increase CF PP-1 activity. Insulin treatment(25 ng/ml) of adipocytes resulted in rapid increase(30-40% over basal levels) in PP-1 activity in the H and PF with a t1/2 of < 1 min and return to basal level in 30 min.Ins effect was dose dependent, with an Ic 50 of 5 ng/ml. Ins treatment did not alter CF PP-1 activity.Trypsin-released PP-1 activity was not affected by Ins suggesting that insulin acts by converting the latent inactive form of PP-1 into active form. Treatment with a cAMP agonist(SpcAMP,10^{-4}) inhibited both PF and CF PP-1 activity.Insulin/SpcAMP did not affect PP-2A levels. The protein contents of 37kD catalytic subunit of PP-1 detected by western blotting with anti PP-1 antibody were not affected by Ins/SpcAMP. Whether the reductions seen in CF/PF PP-1 by SpcAMP are due to site-specific phosphorylation of the putative regulatory subunit with resultant dissociation of 'C'subunit from 'RG'subunit is under invstigation.

BZ 604PTP-1C IS PHOSPHORYLATED ON TYROSINE IN MACROPHAGES IN RESPONSE TO COLONY STIMULATING FACTOR-1, Karen L. Berg, Yee-Guide Yeung, Fiona J. Pixley, E. Richard Stanley, Department of Developmental Biology and Cancer, Albert Einstein College of Medicine, Bronx, NY 10461

The action of the mononuclear phagocyte growth factor, colony stimulating factor-1 (CSF-1) is mediated by a specific tyrosine kinase receptor which is the c-*fms* proto-oncogene product. Incubation of macrophages with CSF-1 causes CSF-1 receptor (CSF-1R) dimerization, activation, and autophosphorylation, followed by the tyrosine phosphorylation of several, primarily cytoplasmic proteins, whose identity has not yet been established. In order to study the role these proteins play in CSF-1R-mediated signal transduction, we have taken the approach of identifying them directly by purification and microsequencing. BAC1.2F5 macrophages were incubated in the absence and presence of CSF-1, homogenized, and the cytosolic fraction subjected to anti-phosphotyrosine affinity chromatography. The phosphotyrosine-containing proteins were then further purified by size-exclusion chromatography and reverse phase HPLC. Tryptic peptides of HPLC-purified proteins from both stimulated and control cells were then sequenced directly. One of proteins, (in the 45-70 kDa range) present only in fractions from CSF-1-stimulated cells, was found to be PTP-1C, the SH2-containing, cytoplasmic protein tyrosine phosphatase. The identity of this protein was confirmed by western blotting with an antibody raised to a unique PTP-1C peptide, and its tyrosine phosphorylation demonstrated by western blotting with an antibody to phosphotyrosine. The rapid, growth factor-induced tyrosine phosphorylation of PTP-1C suggests that it may be involved in very early events in growth factor signal transduction.

BZ 605 ROLE OF TYROSIN-KINASES AND PHOSPHATASES IN PROGRAMMED CELL DEATH, Vincenzo Bronte, Antonio Rosato, Annalisa Zambon, Susanna Mandruzzato, Paola Zanovello and Dino Collavo, Institute of Oncology, University of Padua, Italy.

Eukaryotic cells undergoing programmed cell death (PCD) display a characteristic morphology called apoptosis, and bear oligonucleosome fragments of DNA. Although a sustained increase in intracellular Ca^{2+} has been described, the signal pathway for PCD induction remains to be defined. In fact, it was demonstrated that extracellular ATP (ATP_e) is able to induce Ca^{2+}-independent osmotic cell lysis and DNA fragmentation in numerous cell lines. We observed that the addition of Genistein, a specific inhibitor of protein-tyrosine-kinases (PTK), during incubation of P815 and L1210 cells with ATP_e induces a dose-dependent inhibition of ATP_e-mediated DNA fragmentation without affecting cellular lysis. Analogous findings were obtained following pre-treatment with Tyrphostin or Herbimycin A, two PTK-inhibitors endowed with a mechanism of action different from Genistein. The control of PCD by PTK-inhibitors is not restricted to ATP_e-induced apoptosis as similar results were obtained in CTLL-2 cells cultured in the absence of IL-2, glucocorticoid-treated thymocytes and target cells killed by cytotoxic lymphocytes. Finally, ATP_e modifies the phosphorylation pattern of several proteins in P815 and L1210 cell lines, a few minutes after its addition. As the activity of protein phosphotyrosin phosphatases (PTP) might tightly regulate signal transduction by PTK, we used orthovanadate and phenylarsine oxide to specifically inhibit PTP in intact cells. Surprisingly, these molecules also caused a reduction in apoptosis without affecting cell viability. These results suggest that a complex interaction between PTK and PTP is a crucial event in PCD.

BZ 606 CLONING OF PROTEIN TYROSINE PHOSPHATASES FROM MURINE BRAIN. David Chantry, Tony Hunter. Department of Molecular Biology and Virology, Salk Institute, 10,010 N. Torrey Pines Road, La Jolla, CA 92037.

Protein tyrosine phosphatases (PTPases) are a rapidly growing family of proteins which are thought to be involved in regulating the activity of protein tyrosine kinases. Over 17 mammalian PTPases have now been cloned and the number continues to increase. We have aligned the catalytic domain(s) of these PTPases and used this both to establish phylogenetic relationships between the PTPases and (using highly conserved stretches of amino acids within the catalytic domain) to design primers for use in the polymerase chain reaction, thus allowing the identification of novel PTPases.

Using mouse brain cDNA as a template we have isolated a number of PTPases including the previously cloned PTP1B, TC-PTP, PTPH1, and PTP-MEG2. In addition two novel PCR products were identified and these were used to screen mouse brain cDNA libraries. cDNA clones representing two novel PTPases (designated clone 5.1 and clone 5.18) were isolated. In contrast to many of the currently identified PTPases Clone 5.1 appears to show a highly restricted tissue distribution being expressed only in brain and testes. The significance of this is currently being explored. Both cDNAs *appear to lack sequences* at the 5' end of the gene and we are in the process of isolating full length cDNAs for these PTPases. In addition data will be presented on their enzymatic activity *in vitro* and their *in vivo* expression.

BZ 607 **THE MURINE HOMOLOG TO PTP1C IS EXPRESSED IN MAMMARY EPITHELIAL CELLS AND ASSOCIATES SPECIFICALLY WITH A TYROSINE PHOSPHORYLATED PROTEIN IN VITRO.** David L. Dankort and William J. Muller. The Institute for Molecular Biology and Biotechnology, McMaster University, Hamilton ON, Canada L8S 4K1

The proto-oncogene c-erb-B2, a receptor tyrosine kinase, is found amplified in 10-30% of human breast caricinomas. Moreover, ectopic expression of either a constuitively activated c-erb-B2 murine homolog (*neu**) or polyoma middle T antigen which binds and activates at least three src family tyrosine kinases in the mammary gland leads to transformation of the entire mammary epithelium in trangenic mice. Overexpression of *c-neu* in the mammary epithelium leads to clonal tumor formation although the transgene was expressed throughout the mammary gland. Interestingly, the mean latency of tumor formation in *c-neu* transgenic lines was significantly increased. These results argue that the mammary epithelium is particularly sensitive to the expression of deregulated tyrosine kinases. Because protein tyrosine phosphorylation is regulated by the opposing actions of protein tyrosine kinases and phosphatases (PTPases), we sought to identify PTPases expressed in the mammary gland. One of the genes isolated from a primary screen was found to have a narrow tissue distribution with high levels expressed in the mammary gland as well as the spleen and thymus. Following the cloning and sequencing of this gene, termed PTPM1, it was found that it was homologous to the human non-receptor PTPase, PTP1C. In addition to containing a single PTPase domain, PTP1C contains two src homology 2 (SH2) domains which mediate protein tyrosine phosphorylated protein interactions. An initial screen of proteins which interact with PTPM1, we have utilized a series GST fusion proteins made to the SH2 domains of PTPM1. Data will be presented concerning the expression of PTPM1 in the mammary epithelium as well as preliminary results concerning the associated proteins.

BZ 608 RECEPTOR PROTEIN TYROSINE PHOSPHATASE α: INVOLVEMENT IN NEURONAL DIFFERENTIATION AND REGULATION OF ENZYMATIC ACTIVITY, Jeroen den Hertog, Cornelieke E.G.M. Pals*, Wiebe Kruijer* and Tony Hunter, Molecular Biology and Virology Laboratory, the Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037 and * Hubrecht Laboratory, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands

Protein tyrosine kinases (PTKs) play an important role in cellular proliferation and differentiation. In this respect little is known about their enzymatic counterpart, the protein tyrosine phosphatases (PTPases). We demonstrate that the transmembrane, receptor-like protein tyrosine phosphatase α (RPTPα) can play an important role during neuronal differentiation. RPTPα-mRNA expression is transiently induced during neuronal differentiation of pluripotent embryonal carcinoma (EC) and neuroblastoma cells. Ectopic expression of RPTPα in pluripotent murine P19 EC cells alters the differentiation fate of these cells in favour of neuronal differentiation, as illustrated by the ability of the differentiated derivatives to generate action potentials. By contrast, the differentiation fate of P19 cells expressing a functional mutant of RPTPα which lacks PTPase-activity is unaltered. This demonstrates that RPTPα-activity is essential for the altered differentiation fate, indicating that PTPases like PTKs play an important role in development.

Using stably transfected P19 cells, expressing functional RPTPα, we investigated whether RPTPα-activity is regulated by extracellular stimuli. We demonstrate that RPTPα-activity is transiently enhanced following stimulation of living cells with phorbol ester, due to a two- to three-fold increase in substrate-affinity. Phorbol ester stimulates Ser-phosphorylation of RPTPα with similar kinetics. Enhanced phosphorylation of RPTPα is essential for the increase in activity, since *in vitro* dephosphorylation of RPTPα reduces RPTPα-activity to basal levels. Activation of several receptor PTKs leads to activation of protein kinase C (PKC), the intracellular receptor for phorbol ester. Here we demonstrate that activation of PKC leads to enhanced PTPase-activity, indicating that PTPases may be actively involved in negative feed-back of receptor PTK-signalling.

BZ 609 MODULATION OF T CELL RECEPTOR SIGNAL TRANSDUCTION VIA AN EGF-RECEPTOR/CD45 CHIMERA. Dev M. Desai, Jan Sap*, Joseph Schlessinger*, and Arthur Weiss. University of California, San Francisco School of Medicine and the Howard Hughes Medical Institute, San Francisco, CA 94122. *New York University Medical Center, New York, NY 10016.

CD45 (leukocyte common antigen [LCA], T200, or B220), a transmembrane protein tyrosine phosphatase is expressed on all nucleated cells of hematopoeitic origin. The cytoplasmic region of CD45 is comprised of two invariant phosphatase domains, while the extracellular portion of CD45 exists in multiple isoforms due to alternative splicing of at least 3 exons. The pattern of isoform expression is regulated in a cell activation, developmental and tissue specific manner.

A number of studies have demonstrated the requirement for cell surface expression of CD45 for normal antigen receptor in B and helper and cytotoxic T cells. CD45 negative cells are unable to phosphorylate proteins on tyrosine, flux calcium or generate inositol phospholipids in response to T cell antigen receptor (TCR) stimulation. In addition, evidence has been presented for specific CD45 isoform co-capping with T cell surface molecules such as LFA-1, CD2, CD4 and CD8. Moreover, CD45 has been demonstrated to co-immunoprecipitate with the TCR, Thy-1 and CD2. Since it appears that CD45 can associate with multiple cell surface molecules involved in signal transduction and because such an association may be functionally important, we constructed a chimeric molecule, in which the extracellular and transmembrane domains of CD45 were replaced with that of the epidermal growth factor receptor (EGFR), to determine whether signalling functions could be restored in a CD45 deficient variant of the T cell line HPB.ALL.

Our results demonstrate that the EGFR/CD45 chimera is able to reconstitute signal transduction in CD45 deficient cells to the same degree as wild type CD45. Moreover, we have found that we can now modulate TCR signal transduction using EGF or transforming growth factor a (TGFα), both of which are ligands for the EGFR. The addition of EGF or TGFα concomitantly with stimulatory anti-TCR antibody results in a delayed and blunted increase in $[Ca^{2+}]_i$ levels. Even more surprising is that the addition of EGFR ligand post-TCR stimulation results in a rapid and dramatic decrease in intracellular $[Ca^{2+}]_i$ to basal levels. These results have implications regarding the regulation of CD45 function.

BZ 610 PROTEIN PHOSPHATASES REGULATE CYTOSKELETAL INTERMEDIATE FILAMENT STRUCTURE.
J.E. Eriksson and R.D. Goldman, Dept. of CMS Biology, Northwestern University, 303 E. Chicago Ave. Chicago, IL 60661-3008

In a previous study we showed that protein phosphatase activity is required to maintain a normal vimentin intermediate filament (IF) structure in BHK-21 cells (Eriksson JE, Brautigan, DL, Vallee, R, Olmsted, J and Goldman RD 1992; Proc. Natl. Acad. Sci., in press). Protein phosphatase inhibition with nanomolar doses of calyculin-A (cl-A), a potent inhibitor of the type-1 (PP1) and type-2A (PP2A) serine/threonine protein phosphatases, causes a rapid disruption of the vimentin IF structure in these cells. Further studies have shown that the breakdown of the IF networks is correlated in a dose-dependent fashion with 10-40-fold increases in the ^{32}P-labelling of vimentin. This elevated vimentin phosphorylation is accompanied by increased solubility of vimentin both in the low (6000 x g) and high speed supernatants (200 000 x g) of 1% Triton X-100-extracted IF preparations from cl-A-treated cells. The increases in vimentin phosphorylation takes place at few interphase-specific sites and the $p34^{cdc2}$ mitosis-specific site does not display increased labelling. The rapidly elevated phosphorylation of vimentin, upon protein phosphatase inhibition, indicates that there is a continuous turnover of phosphate on vimentin on interphase-specific sites. These sites are evidently important for maintenance of a normal interphase IF cytoskeletal network, since an alteration of the phosphate equilibrium on these sites causes an immediate modification of the structure and assembly state of vimentin IF. As there is high phosphate turnover on these sites, the regulation of their phosphorylation states may also underlie the mechanisms involved in the exchange between IF protein subunits and polymers, recently elucidated by microinjection experiments (Miller R, Vikstrom K, and Goldman RD 1991; J. Cell Biol. 113: 843-855; Vikstrom K, Lim, S-S, Goldman, RD, Borisy, GG 1992, J. Cell Biol. 118: 121-129). The sequences of these *in vivo* phosphorylation sites, as well as the possible specificity of either PP1 or PP2A for these sites are being determined. Furthermore, we are attempting to establish which kinase(s), in addition to cAMP-dependent kinase, are responsible for the phosphorylation of these interphase-specific sites. Overall, the data available support the intriguing idea that the dynamic properties displayed by intermediate filaments during different phases of the cell cycle, are regulated by an orchestrated activation and deactivation of protein phosphatases and kinases, such as PP1 and $p34^{cdc2}$ Our goal is to define the functional roles of the protein phosphatases and kinases involved in this scheme. Supported by NIH.

BZ 611 INHIBITION OF PROTEIN TYROSINE PHOSPHATASE ACTIVITY IN HER14 CELLS BY MELITTIN AND CALCIUM IONOPHORE A23187. Mourad Errasfa and Arnold Stern, Department of Pharmacology, New York University Medical Center, New York, NY 10016.

Protein tyrosine phosphatases (PTPs) may play a major role in cell signal transduction. In this study, total PTP activity was investigated in NIH 3T3 cells transfected with the human epidermal growth factor (EGF) receptor. Cell homogenate was used to measure PTP activity expressed as a release of free phosphate from either ^{32}P-labeled- Poly(Glu/Tyr)(4:1) or myelin basic protein as substrates. PTP activity was decreased after treatment of cells with melittin or with the calcium ionophore A23187. The effect of both drugs was significantly prevented after chelating extracellular calcium with EGTA. The cyclooxygenase inhibitor indomethacin had no effect on melittin-induced inhibition of PTP activity, however, it increased the inhibition induced by calcium ionophore A23187. Arachidonic acid significantly prevented the decrease of PTP activity that was induced by either melittin or A23187. PGE_2, a cyclooxygenase product of arachidonic acid significantly prevented the A23187-, but not the melittin-induced PTP inhibition.The preexposure of cells to EGF resulted in an enhancement of the melittin-induced inhibition of PTP activity, but no change of the A23187 effect. Phorbol myristate acetate did not effect PTP activity of HER14 cells, however, it significantly reversed the A23187 - but not the melittin-induced inhibition of PTP activity. These results suggest that PTP activity in HER14 cells can be inhibited by calcium influx. This inhibition can be modulated by arachidonic acid, EGF receptor and protein kinase C.

BZ 612PHOSPHATASE INHIBITORS MODULATE THE EXPRESSION OF E-SELECTIN GENE IN HUMAN ENDOTHELIAL CELLS.

P.Ghersa,R. Hooft van Huijsduijnen,J.Whelan,Y.Cambet and J.F.Delamarter, GLAXO Institute for Molecular Biology, Chemin des Aulx, 1228 Plans-les-Ouates, Geneva,Switzerland.

E-selectin is a glycoprotein specifically expressed on activated endothelial cells. Protein phosphorylation has been shown to play a key role in E-selectin gene activation. Here we show that dephosphorylation of specific factors is also implicated in E-selectin gene regulation. Okadaic Acid, an inhibitor of Protein Phosphatases 1(PP1) and 2A (PP2A), increases the levels of E-selectin mRNA induced by cytokine, in a dose dependent manner. E-selectin mRNA is superinducible by inhibition of protein synthesis (Ghersa P. et al.JBC October1992). However, the addition of Okadaic Acid blocks the superinduction of E-Selectin mRNA.These data indicate that protein synthesis is required for the positive effect of Okadaic Acid suggesting that its substrate phosphatase turns over very rapidly. Calyculin A , a more potent inhibitor of Phosphatase 1 and 2A, shows the opposite effect to Okadaic Acid : it decreases the expression of E-Selectin mRNA in a dose dependent manner. We show that activation of NFkB is increased by Okadaic Acid and decreased by Calyculin A. Similarly, in endothelial cells transfected with vectors carrying hybrid E-selectin promoter/CAT reporter gene sequences, CAT activity is enhanced by Okadaic Acid and decreased by Calyculin A. The opposite effects obtained using two phosphatase inhibitors suggest that different phosphatases might be implicated during the induction and down-modulation of the E-Selectin gene.

BZ 613 THE ROLE OF PROTEIN PHOSPHATASES IN THE SUPPRESSION OF THE MAP KINASE SIGNAL TRANSDUCTION PATHWAY: Timothy A.J.Haystead, Department of Pharmacology, Health Sciences Center, University of Virginia, Charlottesville, VA 22908.

Treatment of isolated rat adipocytes or NIH 3T3 cells with insulin, PMA or epidermal growth factor (EGF) results in an acute but transient activation (T1/2 = 2.5 minutes; Tmax = 5 minutes) of p42 and p44 MAP kinases (mapk). Paralleling, but preceding the kinetics of MAP kinase activation and inactivation, is an acute activation (T1/2 = 40 seconds; T max = 90 seconds) followed by rapid inactivation of MAP kinase kinase (activation lost within 4 minutes). Importantly, activation of both MAP kinase kinase and MAP kinase is greatly augmented and sustained in the presence of okadaic acid. These results are consistent with the hypothesis that MAP kinase kinase activates MAP kinase *in vivo* and that both enzymes are activated by increased phosphorylation. We are particularly interested in the means by which these protein kinases become rapidly inactivated and have examined two aspects important in the control of their activity *in vivo*; **1.** the activation time course relationship of protein kinases believed to be involved in the activation of MAP kinase kinase; **2.** the types of cellular protein phosphatases which may inactivate $p42^{mapk}$, MAP kinase kinase and Raf 1 kinase *in vivo*.

1.Our first approach was to examine the time course kinetics of cRAF protein kinase activation in relation to that of MAP kinase kinase. Several laboratories have demonstrated that transfection of cells with vRAF results in the activation of both MAP kinase kinase and MAP kinase. These findings implicate MAP kinase kinase as the activator of MAP kinase kinase. Using rapid freeze clamping techniques, following insulin and PMA treatment of adipocytes, we have measured the activation kinetics of cRAF in relation to that MAP kinase kinase and MAP kinase. **2.** In conjunction with these studies we report the use of a recombinant $p42^{mapk}$ protein phosphatase substrate that distinguishes two distinct protein phosphatases which appear to regulate the dephosphorylation of MAP kinase *in vivo*.

BZ 614 SEARCH FOR PROTEIN-TYROSINE PHOSPHATASES INVOLVED IN EPIDERMAL GROWTH CONTROL

Wiljan Hendriks, Jan Schepens, Patrick Zeeuwen, Candida van Hooijdonk*, Paul Mier* & Bé Wieringa, Dept. of Cell Biology & Histology and *Dermatology, Univ. of Nijmegen, P.O.Box 9101, NL-6500 HB Nijmegen, The Netherlands.

Tyrosine phosphorylation, a key regulatory mechanism in growth factor signal transduction, neoplastic transformation and cell cycle progression, is determined by the opposing action of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPases). To investigate a possible involvement of PTPases in cell growth-related skin diseases such as psoriasis, we analyzed PTPase expression patterns in a murine cell line, Balb/MK keratinocytes whose growth characteristics can be modulated in a controlled fashion.

RNA isolated from resting or growing Balb/MK cells was used as template in an RT-PCR reaction containing a degenerate primer pair based on conserved amino acid residues within PTPase catalytic domains. The resulting PCR fragments were used as a probe on dot-blots containing equimolar amounts of ten different murine PTPase clones previously isolated from a brain tissue cDNA library (Schepens et al. 1992 Mol. Biol. Rep. in press; Hendriks et al., unpublished). In addition, Northern blot analysis was performed using individual PTPase cDNA clones as probes.

No expression in keratinocytes was observed for the murine homologs of human HPTPβ and HPTPδ, and for PTP13 and PTP38, two novel PTPases identified at our laboratory. For the murine homologs of human PTP1B, BPTP-1, LAR, HPTPα, and HPTPϵ expression was observed at similar relative amounts in resting and cycling keratinocytes. A third novel PTPase identified by us, PTP14, was the most prominent PTPase expressed in the Balb/MK cells. This major keratinocyte PTPase, encoded by a 9 kb mRNA, is also weakly expressed in some other tissues. The structural analysis of the PTP14 mRNA product is currently in progress. Further characterization of quantitative or qualitative changes of the PTP14 protein might provide insight into the function of this PTPase in epidermal growth control.

BZ 615 CHARACTERIZATION OF SERINE / THREONINE PROTEIN PHOSPHATASE INHIBITORS, Richard E. Honkanen, Burt A. Codispoti, Kathy Tse, and Alton L. Boynton. Department of Cell and Molecular Biology, Pacific Northwest Research Foundation, Seattle, WA 98122.

The phosphorylation of biologically active proteins is currently recognized as an important mechanism of regulating a variety of cellular events. Assuming protein phosphorylation initiates an event, the return to a basal state then requires dephosphorylation or the degradation and resynthesis of a protein in an unphosphorylated form. Recently a variety of potent and specific inhibitors of serine/threonine protein phosphatases (okadaic acid, microcystins, nodularin, calyculin A, and tautomycin) have been identified, all of which potently inhibit the activity of serine/threonine protein phosphatases types 1 (PP1), 2A (PP2A) and 3 (PP3). Thus, a considerable amount of interest has evolved around the use of these compounds as tools to determine the intracellular functions of serine/threonine protein phosphatases. However, there appears to be a considerable amount of confusion as to the concentrations at which these compounds are effective inhibitors of PP1, PP2A and PP3.

This study compares the inhibitory effects of okadaic acid, structurally modified forms of okadaic acid, microcystins, nodularin, calyculin A, and tautomycin on the activity of the purified catalytic subunits of PP1, PP2A and PP3. Under identical experimental conditions, okadaic acid, microcystin-LR, and microcystin-LA were found to be more potent inhibitors of PP2A and PP3 than PP1 (order of potency PP2A > PP3 > PP1). Nodularin inhibits the activity of PP1 and PP3 at about the same concentration which is slightly higher than that required to inhibit PP2A. Calyculin A and tautomycin were equally effective at inhibiting the activity of PP1, PP2A and PP3. For PP2A the order of potency was Microcystin-LR = Microcystin-LA = Nodularin > calyculin A > okadaic acid > tautomycin. The stability and cell permeability of these compounds will be discussed.

BZ 616 EXPRESSION CLONING OF A NOVEL HUMAN DUAL SPECIFICITY PHOSPHATASE,

Toshio Ishibashi, Donald P. Bottaro, Andrew M.-L. Chan, Toru Miki and Stuart A. Aaronson, Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD 20892

Using an expression cloning strategy, we isolated a cDNA encoding a human protein tyrosine phosphatase (PTP). Bacteria expressing the kinase domain of the KGF receptor (*bek* /FGFR-2) were infected with a phagemid expression library and screened with a monoclonal anti-phosphotyrosine antibody (anti-pTyr). Among several clones showing decreased anti-pTyr recognition, one displayed phosphatase activity toward the kinase *in vitro*. The 4.1 kb cDNA encodes a deduced protein of 185 amino acids with limited sequence similarity to vaccinia virus phosphatase (VH1). The purified protein expressed in bacteria dephosphorylated several activated growth factor receptors as well as serine-phosphorylated casein *in vitro*. Both serine and tyrosine phosphatase activities were completely abolished by mutagenesis of a single cysteine residue conserved in VH1 and the VH1-related (VHR) human protein. These properties suggests that VHR is capable of regulating intracellular events mediated by both tyrosine and serine phosphorylation.

BZ 617 EXPRESSION OF THE CYTOPLASMIC DOMAIN OF THE TYROSINE PHOSPHATASE, CD45, IS SUFFICIENT FOR T CELL RECEPTOR MEDIATED SIGNAL TRANSDUCTION, Gary A. Koretzky, Robin Hovis, Susan Ross, and Jerald Donovan. Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA 52242

CD45 is a cell surface tyrosine phosphatase found on all nucleated hematopoietic cells. Recently we and others have demonstrated that T cell antigen receptors (TCR) on T cells deficient in CD45 expression are uncoupled from their normal signal transduction machinery. Reconstitution of CD45 expression by gene transfer of wild type CD45 cDNA rescues TCR-mediated signal transduction in our CD45-deficient variants. Although not yet proven, it is speculated that the role of CD45 is to dephosphorylate a critical site, perhaps the regulatory tyrosine found on the lck tyrosine kinase (TK). It remains unclear which features of the CD45 molecule are essential in the regulation of TCR signaling. It is possible, for example, that the complex extracellular domain of CD45 with its various isoforms is critical but also possible that only the cytoplasmic domain (conserved among all CD45 isoforms) is required. We have addressed this by generating a chimeric molecule containing the extracellular and transmembrane domains of an allele of human class I MHC and the cytoplasmic domain of CD45. cDNA encoding the chimera was transfected into a CD45-deficient clone and high level expressors were isolated. Stimulation of the TCR on the cells reconstituted with the chimera resulted in the generation of the expected second messengers including the activation of a TK, phosphorylation of phospholipase Cγl, and the generation of phosphatidylinositol-derived second messengers. Additionally, immunoprecipitates of the chimeric molecule were found to possess kinase activity in in vitro assays. These data suggest that the cytoplasmic region of CD45 contains all of the information necessary for the TCR to transduce its known signals. We are currently evaluating the ability of the chimera to directly bind lck. Additional cDNA constructs have been made deleting increasingly large regions of the CD45 cytoplasmic domain. Once expressed in our cells, these mutant proteins will enable us to define better the minimal sequence of CD45 required for TCR signaling and lck binding.

BZ 618MOLECULAR CLONING OF A SECOND MAMMALIAN SH2 CONTAINING TYROSINE PHOSPHATASE,

Robert J. Lechleider, Robert M. Freeman, Jr., Benjamin G. Neel, Molecular Medicine Unit, Beth Israel Hospital, Boston, MA, 02215.

Src homology 2 (SH2) domains are sequences found in a variety of cellular signaling molecules which direct binding to specific phosphotyrosyl proteins. Recently our lab and others cloned the first member of the SH2 containing phosphatase family, which we named SH-PTP1. We report the cloning and characterization of a second mammalian SH2 containing phosphatase, human SH-PTP2. SH-PTP2 contains two SH2 domains, is expressed ubiquitously, has a complex genomic organization, and has been highly conserved during evolution. SH-PTP2 expressed in bacteria is an active protein tyrosine phosphatase. Sequence comparison and similarity of expression suggest that SH-PTP2 is the human homolog of the Drosophila gene *corkscrew*, which acts in concert with D-*raf* to transduce the signal generated by the tyrosine kinase *torso*. Using glutathione S-transferase fusion proteins, we generated rabbit antibodies to the C-terminal portion of SH-PTP2. These antibodies recognize a 68 kD protein by both immunoprecipitation and western blotting. Studies exploring the association of SH-PTP2 with cellular phosphotyrosyl proteins and the effects of these associations on SH-PTP2 activity will be reported.

BZ 619 SIGNALLING AND FUNCTION OF THE SH2 DOMAIN CONTAINING TYROSINE PHOSPATASE SHPTP1

Ulrike Lorenz, Jorge Plutzky, Pamela Beahm, Sharon Chang, and Benjamin G. Neel.
Molecular Medicine Unit, Beth Israel Hospital, Boston, MA 02215.

Previously, we and others described the cloning of a novel non-transmembrane phosphotyrosine phosphatase containing two SH2 domains (SHPTP1). We have raised and affinity-purified polyclonal antibodies to a glutathione S-transferase-SHPTP1 fusion protein (GST-SHPTP1). Indirect immunofluorescence studies of endogenous SHPTP1 in human and murine hemato-poietic cells as well as of SHPTP1 transiently overexpressed in HeLa cells reveal that this protein is cytoplasmic.

Immunoprecipitations identify SHPTP1 as a 65 kDa phospho-protein. Phosphorylation is predominantly on serine. Additional phosphorylation on tyrosine is detectable in cells which express activated forms of several src family kinases, as well as in LSTRA cells, which overexpress the src-like kinase lck. Ongoing experiments will determine whether SHPTP1 is a direct substrate of these kinases and whether tyrosine phosphorylation changes its phosphatase activity. Moreover, when anti-SHPTP1 immuno-precipitates are prepared under mild conditions from LSTRA cells, a tyrosyl phosphoprotein of about 160 kDa co-precipitates with SHPTP1. Experiments using various deletions of the GST-SHPTP1 fusion protein show that the more amino-terminal SH2 domain is sufficient to bind the 160 kDa protein. The 160 kDa LSTRA protein is not GAP, PLCγ-1, or p190 but further studies are necessary to clarify the identity of this protein. Contrary to the results of the LSTRA cell line, a more complex pattern of several higher molecular weight phosphotyrosyl proteins co-immuno-precipitates with SHPTP1 in the IL3-dependent murine pre-B cell line BaF3. This pattern changes with time following IL3 stimulation of factor-deprived cells. Studies are in progress to determine whether these proteins form complexes or share epitopes with SHPTP1. Taken together, our data suggest that SHPTP1 may play a role in terminating and/or transmitting early tyrosine phosphorylation events following growth factor stimulation of hematopoetic cells.

BZ 620 CLONING AND SEQUENCE ANALYSIS OF A NOVEL cDNA ISOLATED FROM HUMAN INSULINOMA THAT ENCODES A PUTATIVE PROTEIN TYROSINE PHOSPHATASE, Jia Lu, Yasuhiro Goto, Abner Louis Notkins and Michael S. Lan, Laboratory of Oral Medicine, National Institute of Dental Research, National Institutes of Health, Bethesda, MD 20892

A novel 3.6 kb cDNA, IA-2, with a 2937 bp open reading frame was isolated from a human insulinoma subtraction library (ISL-153). The predicted amino acid sequence of IA-2 revealed a 979 amino acid protein with a pI value of 7.09 and a molecular mass of 105,847 daltons. The protein sequence is consistent *with a signal* peptide, an extracellular domain, a transmembrane region and an intracellular domain. The extracellular domain contains an unusual cysteine-rich region following the signal peptide. The intracellular cytoplasmic domain of IA-2 possesses highly conserved regions similar to the catalytic domains found in members of the protein tyrosine phosphatase (PTP) family. Northern blot analysis showed that IA-2 mRNA was expressed in five of five freshly-isolated human insulinomas and a glucagonoma as well as glioblastoma and neuroblastoma cell lines. IA-2 mRNA also was detected in normal brain. It was not found, however, in other normal tissues including lung, heart, liver, kidney, colon, stomach, small intestine, pancreas, and spleen, nor was it found in a variety of other tumor cell lines. The structural features and restricted tissue distribution of IA-2 suggest that it may represent a new member of the receptor-type PTP family with a distinct extracellular domain.

BZ 621 INHIBITION OF TYROSINE DEPHOSPHORYLATION BY A CATALYTICALLY INACTIVE PROTEIN TYROSINE PHOSPHATASE, Kim L. Milarski, Zhong-Yin Zhang, Jack E. Dixon and Alan R. Saltiel, Department of Signal Transduction, Parke-Davis, and Department of Biochemistry, University of Michigan, Ann Arbor, MI 48105.

Protein tyrosine phosphorylation is involved in many cellular processes including regulation of the cell cycle, and the response of cells to mitogens. The amount of protein tyrosine phosphate in cells is controlled by the balance between protein tyrosine kinases and protein tyrosine phosphatases (PTPases). While PTPases can be either cytosolic or transmembrane proteins, all share a conserved catalytic domain. Within this region are an essential cysteine and histidine, both required for catalytic activity. In addition, the catalytic domain contains sequence motifs similar to those found in SH2 domains, which also bind tyrosine phosphorylated proteins. These sequences shared by SH2 domains and PTPases appear to be involved in binding tyrosine phosphate. We have found that a PTPase mutated at the essential cysteine, while catalytically inactive, still binds tyrosine phosphate and can act as a dominant negative inhibitor of dephosphorylation by an active PTPase. Like SH2 domains, the inactive PTPase protects substrates from dephosphorylation. Preliminary results suggest that the mutant phosphatase is more efficient than a variety of SH2 domains at protecting substrates from dephosphorylation.

BZ 622 **Mitotic initiation is regulated by a family of phosphotyrosine phosphatases in fission yeast.** Jonathan B.A. Millar and P. Russell, Department of Molecular Biology, MB3, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037.

We have previously shown that the fission yeast cdc25^{+} gene encodes a phosphotyrosine phosphatase (PTPase) that dephosphorylates tyrosine-15 of p34^{cdc2} to activate the p34^{cdc2}/cyclin B complex and cause mitotic initiation. We have identified a new gene, pyp3^{+}, by its ability when cloned on a multicopy plasmid to suppress a temperature sensitive cdc25^{ts} mutation. The pyp3^{+} gene, which encodes for a 33kDa PTPase, is required for viability of the cell in the absence of cdc25 activity. When produced as a fusion protein in bacteria, pyp3-GST efficiently tyrosine dephosphorylates and activates the p34^{cdc2} mitotic kinase *in vitro*. Together this suggests that pyp3^{+} acts in conjunction with cdc25^{+} to dephosphorylate p34^{cdc2} to maintain the correct timing of mitotic initiation. In direct contrast, loss of pyp1^{+} gene function causes an advancement in the timing of mitotic onset by counteracting cdc25^{+} and pyp3^{+} activity and this can be attributed to modulation of the activity of the mitotic inhibitory p107^{wee1} kinase. In collaboration with K.L. Guan and J. Dixon (Univ. of Michigan), we have isolated at third PTPase, pyp2^{+} that is not only structurally related to pyp1^{+} but is also involved in negatively regulating mitotic initiation. The pyp1^{+} and pyp2^{+} PTPases share an essential overlapping function. This demonstrates that non-transmembrane PTPases have not only have different substrates but directly opposing actions on growth regulation, and in particular the timing of mitotic initiation. A model illustrating these findings will be presented. Considering the high evolutionary conservation of mitotic control we consider it likely that homologs of the pyp1, pyp2 and pyp3 PTPases exist in other eucaryotic cells.

BZ 623 BIOCHEMICAL CHARACTERIZATION OF HUMAN Ca^{2+}-SENSITIVE CYTOSOLIC PHOSPHOLIPASE A_2 PRODUCED IN BACULOVIRUS-INFECTED INSECT CELLS. James R. Miller, Robert M. Ellis, Andrew I. Louis, Jennifer S. Small, Tamara K. Wyrick, Ruth M. Kramer, and Gerald W. Becker. Departments of Biotechnology and Pulmonary Research, Lilly Research Laboratories, Indianapolis, IN 46285.

The cDNA for human cytosolic phospholipase A_2 (cPLA$_2$) has been cloned into a baculovirus expression vector for the production of cPLA$_2$ in insect tissue culture cells where optimal production reached ~50 mg per liter. The recombinant protein migrates on SDS-PAGE as a 110 kDa band in a manner identical to authentic protein from human U937 leukemia cells. Identification and characterization of biologically active recombinant cPLA$_2$ are based on several criteria. This protein, purified to near homogeneity from insect cells, showed the same Ca^{2+} activation profile as authentic cPLA$_2$. Biochemical characterization by HPLC/Electrospray Mass Spectrometry suggests that the initiating formylmethionine is removed and the N-terminal serine is modified by an acetyl moiety; purified cPLA$_2$ from U937 cells is blocked at the N-terminus. Phosphoamino-acid analysis of cPLA$_2$ from ^{32}[P$_i$]-labeled insect cells determined that the protein was specifically labeled on serine residues as previously shown for cPLA$_2$ from CHO cells. Peptide analysis is underway to determine the site(s) of phosphorylation.

BZ 624 O-PHOSPHO-L-TYROSINE INHIBITS CELLULAR GROWTH BY ACTIVATING PROTEIN TYROSINE PHOSPHATASE, Mishra, S. and Hamburger, A.W., Division of Cell and Molecular Biology, University of Maryland Cancer Center, Baltimore, MD 21201

We report that incubation of human renal and breast carcinoma cells with O-phospho-L-tyrosine (P-Tyr), a substrate for a wide range of protein tyrosine phosphatases (PTPases), resulted in growth inhibition of these cells in the S phase of the cell cycle accompanied by a decreased accumulation of cyclin proteins A & B as well as $p34^{cdc2}$. P-Tyr incubation led to activation of cellular PTPases resulting in the inhibition of tyrosine phosphorylation of epidermal growth factor receptor (EGFR) as well as of $p34^{cdc2}$. P-Tyr synergistically sensitized the renal carcinoma ACHN cells to killing by chemotherapeutic agents doxorubicin and etoposide. These growth inhibitory properties of P-Tyr in vitro suggests its possible use as an anti-cancer agent.

BZ 625 REGULATION OF HEAT SHOCK RESPONSE BY ser/thr AND tyr- PHOSPHATASES. Nahid F. Mivechi, Toshimi Murai, and George M. Hahn. Dept. Of Radiat. Oncology, Stanford Univ. School of Med., Stanford, CA, 94305.

Previousely we showed that purified mammalian HSP-70 activates ser/thr phosphatases(PP1)in vitro. Such results prompted us to investigate the possibility of negative regulation of HSP-70 on the heat shock transcription factor (HSF)following heat. After heat shock, HSF is known to be phosphorylated, and bind to the heat shock element(HSE). Our hypothesis, therefore, was that HSP-70 accumulation in cells after heat may result in PP1 activation and thereby facilitate inactivation of phosphorylated HSF. We now show that Okadaic Acid(OA), a ser/thr phosphatase inhibitor causes an increase in activated HSF as measured by gel retardation assays. Such an increase in activated HSF also results in an increase in synthesis of β-galactosidase in a CHO cell line (HB-43) containing the heat shock promoter fused to the β-galactosidase gene. We have also studied the effect of sodium vanadate, a tyr-phosphatase inhibitor on HSF activation and β-galactosidase induction in HB-43 cells. Preincubation of cells with doses of vanadate that caused no cytotoxicity, reduced HSF activation and binding to the HSE after heat shock and virtually abolished β-galactosidase induction. Reduced HSP synthesis was confirmed by SDS-PAGE and western blot analysis using antibodies to HSP-70 and HSP-28.

From these results we postulate that the increase in HSP-70 after heat shock may activate ser/thr phosphatases. These are likely to be involved in dephosphorylation of HSF. Further, we also postulate that tyr-phosphatases are involved in regulating HSF activation and most likely play an important role in the early pathway of heat shock signal transduction.

BZ 626 CHARACTERIZATION OF THE MURINE INTRACELLULAR PHOSPHOTYROSINE PHOSPHATASE, Bedrich Mosinger, Jr. and Heiner Westphal, Laboratory of Mammalian Genes and Development, Bldg.6B, Rm.215, NICHD/NIH, Bethesda, MD 20892.

Phosphotyrosine phosphatases (PTP) are enzymes catalyzing the removal of phosphate specifically from tyrosine residues in proteins. Two distinct types of PTP can be distinguished, one that has structural feature of a membrane receptor and the second that is intracellular. The functions of most of these PTP are still unknown, however their expected role in the regulation of tyrosine phosphorylation suggests that they may be involved in signal transduction, cell growth and proliferation, oncogenesis and ontogenesis.

We have isolated and characterized a murine cDNA (MPTP) coding for intracellular PTP, a homologue of the human T-cell PTP. The MPTP cDNA encodes a 382 amino acid protein which differs from the human homologue at its carboxy terminus. The MPTP gene is expressed as a 1.9 kb message throughout development and in all adult tissues that we examined.

Current studies are aimed at further characterization of the MPTP gene and at understanding its function. The results from various approaches including mapping studies, overexpression of the MPTP in tissue culture cells and in transgenic animals will be presented.

BZ 627 CALCINEURIN (PROTEIN PHOSPHATASE-2B) IS THE FK-506-SENSITIVE COMPONENT OF THE T CELL SIGNAL TRANSDUCTION PATHWAY, S.J. O'Keefe*, J.N. Parsons*, B. Frantz-Wattley*, E. Nordby*, M.J. Tocci*, J. Tamura†, R.L. Kincaid† and E.A. O'Neill*; *Dept. of Molecular Immunology, Merck Research Labs, Rahway, NJ 07065 and †Immunology Sect., NIAAA/NIH, Rockville, MD 20852.

T-cell proliferation requires induction of interleukin-2 (IL-2) gene transcription via signal transduction pathways which involve activation of protein kinase C and a rise in intracellular Ca^{2+}. The immunosuppressants, Cyclosporin A (CsA) and FK-506, selectively inhibit a Ca^{2+}-dependent pathway. The complexes of either FK-506 or CsA and their respective intracellular binding proteins associate with and inhibit the calmodulin-dependent protein phosphatase, calcineurin, *in vitro*. To assess the relevance of this observation to T cell activation and immunosuppression, *in vivo*, we transfected plasmids that encoded either a wild type or a truncated form of the Gene 1 (murine) or Gene 2 (human) calcineurin catalytic subunit together with a reporter construct containing the IL-2 promoter fused to the chloramphenicol acetyltransferase (CAT) gene into the human leukemic T cell line, Jurkat. Transfection of a catalytic subunit increased the IC_{50} of FK-506, indicating that the interaction of FK-506/FKBP-12 and calcineurin observed *in vitro* also occurs *in vivo*. Most importantly, either wild type catalytic subunit increased IL-2 promoter activity in the presence of PMA and ionomycin and either truncated subunit replaced the required Ca^{2+} signal and acted in synergy with PMA to activate the IL-2 promoter. Cotransfection of a catalytic subunit along with the regulatory calcineurin B subunit increases the IC_{50} of FK-506 over two orders of magnitude but has no effect on the level of IL-2 promoter activation in the presence of PMA compared to transfection of the catalytic subunit alone. These results demonstrate that the heterodimer binds the FK-506/FKBP complex and that at least one other non-calcineurin, Ca^{+2}-dependent signalling pathway is required for maximal promoter activity. Our observations provide the first biological evidence that this phosphatase regulates transcription and suggest a widespread role for calcineurin in the regulation of other immunosuppressant-sensitive processes.

BZ 628 A GENE ENCODING A PUTATIVE PROTEIN TYROSINE PHOSPHATASE SUPPRESSES LETHALITY OF AN N-END RULE-DEPENDENT MUTANT, Irene M. Ota and Alexander Varshavsky, Division of Biology, California Institute of Technology, Pasadena, CA 91125

The N-end rule relates the *in vivo* half-life of a protein to the identity of its N-terminal residue. Distinct versions of the N-end rule operate in all organisms examined. With one exception (RNA polymerase of the Sindbis virus), no physiological substrates of the N-end rule pathway have been identified thus far in either bacteria or eukaryotes. In *S. cerevisiae*, deletion of the *UBR1* gene, which encodes the recognition component of the N-end rule pathway, inactivates this pathway but does not result in an otherwise conspicuous phenotype. In a search for the functions and physiological substrates of the N-end rule pathway, we used a "synthetic lethal" screen to identify yeast mutants whose viability depends on the presence of the *UBR1* gene. Thus far, one such mutant, *sln1* (for synthetic *l*ethal of the *N*-end rule pathway) has been isolated. We cloned a gene, *PTP2*, that complements the *sln1* mutation. *PTP2* encodes a protein with strong sequence similarities to known protein tyrosine phosphatases (PTPases). The *PTP2* gene is unlinked to the *SLN1* gene (which remains to be cloned), and is thus an extragenic suppressor of *sln1*. Deletion of the *PTP2* gene results in a growth defect but does not render cells dependent on the presence of the N-end rule pathway. The tyrosine phosphatase activity of *PTP2* appears to be required for suppression of the *sln1* phenotype since mutation of the putative active site region of Ptp2 is unable to complement *sln1*. Since it is possible that Sln1 is also a PTPase, the *PTP1* and *MIH1* genes that encode two known PTPases of *S. cerevisiae* other than *PTP2* were tested for their ability to complement *sln1*. Neither gene complemented the *sln1* defect. In a more recent attempt to clone *SLN1*, we isolated eight high copy suppressors of *sln1* whose analysis is underway. Our aim is to understand the mechanics and function of a connection between the N-end rule, PTPases and functions essential for cell viability.

BZ 629 THE DROSOPHILA *corkscrew* GENE ENCODES A PUTATIVE PROTEIN TYROSINE PHOSPHATASE THAT FUNCTIONS TO TRANSDUCE THE TERMINAL SIGNAL FROM THE RECEPTOR TYROSINE KINASE *torso*, Lizabeth A. Perkins, Inger Larsen* and Norbert Perrimon*, Department of Genetics,*Howard Hughes Medical Institute, Harvard Medical School, 200 Longwood Ave., Boston, MA 02115

We will describe the characterization of the *Drosophila* gene, *corkscrew* (*csw*), which is maternally required for normal determination of cell fates at the termini of the embryo. Determination of terminal cell fates is mediated by a signal transduction pathway which involves a receptor tyrosine kinase, *torso*, a serine/threonine kinase, *D-raf*, and the transcription factors, *tailless* and *huckebein*. Double mutant and cellular analyses between *csw*, *torso*, *D-raf*, and *tailless* indicate that *csw* acts downstream of *torso* and in concert with *D-raf* to positively transduce the *torso* signal, via *tailless*, to downstream terminal genes. The *csw* gene encodes a putative non-receptor protein tyrosine phosphatase covalently linked to two N-terminal SH2 domains, which is similar to the mammalian PTP1C and SHPTP2 proteins.

BZ 630 THE ROLE OF CD45 TYROSINE PHOSPHATASE ISOFORMS IN SIGNAL TRANSDUCTION IN HUMAN T CELL SUBSETS Anne T. Robinson & Denis R. Alexander Department of Immunology, Institute of Animal Physiology and Genetics Research, Babraham, Cambridge, CB2 4AT.

Differential splicing of exons A,B and C generates eight isoforms of the transmembrane phosphatase CD45. Human T lymphocytes can be separated into functionally distinct $CD45RO^{hi}CD45RA^{lo}$ ($CD45RO^+$) and $CD45RA^{hi}CD45RO^{lo}$ ($CD45RA^+$) subsets. $CD45RO^+$ cells proliferate to a greater extent than $CD45RA^+$ cells in response to recall antigen or mitogenic antibodies. The present study aimed to investigate whether specific CD45 tyrosine phosphatase isoforms affect the efficiency of coupling of the T cell antigen receptor complex (TCR/CD3) to intracellular signals thus explaining the greater proliferative response of $CD45RO^+$ cells. The expression of cell surface activation markers was found to be higher in the $CD45RO^+$ subset, implying that it contains recently activated cells. CD3- and CD2-mediated diacylglycerol (DAG) production were similar in both subsets, indicating that coupling of the CD3 and CD2 antigens to this signalling pathway is not altered by the expression of CD45 exon A. However, mean basal DAG levels were 60% higher in the $CD45RO^+$ subset than in the $CD45RA^+$ subset. CD3-induced protein kinase C (PKC) activation was found to be 140% higher in the $CD45RO^+$ subset. The CD3-induced rise in $[Ca^{++}]_i$ was 80% higher in the $CD45RO^+$ subset whereas the CD2-induced signals were comparable in this respect. We conclude that the greater proliferative response of $CD45RO^+$ cells to CD3 mAbs is a result of the greater CD3-induced rise in $[Ca^{++}]_i$ and PKC activity. This is not necessarily due to the greater efficiency of TCR coupling in $CD45RO^+$ cells, but may reflect the 'primed' status of cells no longer in G_0, in which thresholds for activation are more readily attainable due to higher basal levels of DAG.

BZ 631 STIMULATORY EFFECTS OF THE PROTEIN TYROSINE PHOSPHATASE INHIBITORY, PERVANADATE, ON T-CELL ACTIVATION EVENTS, J. Paul Secrist†, Leigh Ann Burns*, Larry Karnitz*, and Robert T. Abraham†*, Depts. of Pharmacology† and Immunology*, Mayo Clinic, Rochester, MN 55905

T-cell antigen receptor (TCR) ligation triggers a pleiotropic cellular activation response that includes lymphokine secretion, cell-cycle progression, and ultimately, T-cell proliferation. Two important early events in T-cell activation are protein tyrosine phosphorylation and phospholipase C-mediated phosphoinositide hydrolysis. Previous studies have demonstrated that the vanadyl hydroperoxide complex (pervanadate) is a powerful inhibitor of protein tyrosine phosphatase (PTP) activities in several cell lines. In this study, we show that treatment of the human leukemic T-cell line, Jurkat, with pervanadate rapidly induces a series of protein tyrosine kinase-mediated signals that bear a striking resemblance to those induced by TCR-dependent stimuli. Exposure of intact cells to pervanadate stimulated the *in vitro* catalytic activities of both $p59^{fyn}$ and $p56^{lck}$, *src*-family kinases strongly implicated in TCR-mediated signaling. Finally, pervanadate, like TCR crosslinkage, induced interleukin-2 gene expression in Jurkat cells. Although pervanadate treatment inhibited the PTP activity of CD45, the ability of this agent to activate a CD45-negative Jurkat variant indicated that inhibition of a PTP(s) other than CD45 may be responsible for the observed increases in protein tyrosine phosphorylation. Finally, using pervanadate as a TCR-independent stimulus, we have begun to characterize proteins which undergo tyrosine phosphorylation in response to TCR crosslinkage. These studies indicate that pervanadate represents a useful tool in studying protein tyrosine phosphorylation-dependent signaling cascades in T cells and other cell types.

BZ 632 Regulation of serine/threonine protein phosphatases by second messengers in RINm5F insulinoma cells.

Å. Sjöholm[1,2], R. Honkanen[2,3], P.-O. Berggren[1]
1) Dept of Endocrinology, Karolinska Hospital, Box 60500, S-10401 Stockholm, Sweden. 2) University of Hawaii Cancer Research Center, Molecular Oncology Program, 1236 Lauhala Street, Honolulu, HI 96813.

Changes in protein phosphorylation occurs as a result of stimulation of hormone secretion. Stimulation of phosphorylation by protein kinases is relatively well characterized, but much less is known about protein phosphatases that regulate dephosphorylation. Most protein dephosphorylation reactions are catalyzed by type 1 and 2A serine/threonine phosphatases (PPases). We studied PPase regulation in RINm5F insulinoma cells. Addition of prostaglandins, cAMP or cGMP to cell homogenates failed to affect PPase activity. ATP caused 50 % inhibition of PPase-1 activity at 1 mM and PPase-2A at 0.1 mM, while ADP was less potent and AMP and adenosine were inactive. Of inositol polyphosphates, IP_6 produced 50 % inhibition of PPase-1 activity at 6 μM and PPase-2A at 2 μM. Ca^{2+} and TPA slightly elevated PPase-2A activity, while Ca^{2+} suppressed PPase-1. Polyamines suppressed PPase-1 (spermine>spermidine>putrescine), while having no consistent effects on PPase-2A. When PPases were assayed after giving TPA or forskolin + IBMX to intact cells, no changes were detected. Possible effects of other secretagogues are being investigated. We conclude that RINm5F cell PPases are regulated *in vitro* by certain intracellular second messengers and suggest that PPases may be as important as protein kinases in regulation of phosphorylation.

BZ 633 REGULATION OF THE PROTEIN-TYROSINE PHOSPHATASE, CD45, BY PHOSPHORYLATION ON BOTH SERINE AND TYROSINE, David R. Stover and Kenneth A. Walsh, Department of Biochemistry, University of Washington, Seattle, WA 98195

The time course of phosphorylation of CD45 as a result of T cell activation was monitored by immunoprecipitation and phosphoamino acid analysis. CD45 appears to be phosphorylated by at least two kinases as it is rapidly phosphorylated on tyrosine (< 30 seconds) and slowly phosphorylated on serine (~5 minutes). Ten minutes after treatment of T cells with phytohemagglutinin, CD45 is found to be phosphorylated at 4 sites, each of which displays a consensus sequence for casein kinase II (CKII).

In vitro, simultaneous phosphorylation with CKII and v-abl or $p56^{lck}$ causes approximately a 3 fold activation. This increase in activity occurs toward one phosphorylated substrate (RCML) but not toward two other substrates, including the autophosphorylation site of $p56^{lck}$. Phosphorylation by any of these kinases, independently, does not affect CD45 activity, even when ATPγS is used as the donor (to prevent autodephosphorylation by the protein-tyrosine phosphatase). However, When CD45 is first phosphorylated by a tyrosine kinase (using ATPγS), then by CKII, an even greater activation toward RCML is observed (>7 fold). Surprisingly, this activation is not observed after phosphorylation by the same kinases in reverse order. Although the order of phosphorylation is critical, prior phosphorylation by either of the kinases does not appear to alter the location or stoichiometry of phosphorylation by the other kinase. These observations suggest that at least two independent kinases are responsible for cooperatively regulating CD45 activity during the response of a T cell to antigen. We propose that CD45 may be active toward some substrates during the resting phase of T cells, but when activated by phosphorylation, the specificity of CD45 is altered so that alternative or additional substrates are phosphorylated. It is not yet clear whether this occurs by altering the specificity of previously active domains or by switching on a domain that is inactive in resting cells.

BZ 634 PRL-1, A NEW TYPE OF NUCLEAR/CYTOPLASMIC PROTEIN TYROSINE PHOSPHATASE INDUCED IN THE GROWTH RESPONSE Rebecca Taub, Robert H. Diamond, Drew E. Cressman, and Thomas M. Laz Department of Genetics, Howard Hughes Medical Institute, and Division of Gastroenterology, Department of Medicine, University of Pennsylvania School of Medicine, Phila., PA 19104-6145

Control of the phosphorylation state of cellular proteins is critical for normal cellular growth. Immediate-early genes, which are induced by mitogens in the absence of de novo protein synthesis, also play an important role in the regulation of cell growth. Previously, we identified novel immediate-early genes induced in regenerating liver, a physiologically normal model of cellular proliferation. One of these genes, *PRL-1* was of interest because it is induced in mitogen-stimulated cells and regenerating liver, but constitutively expressed in insulin-treated rat H35 hepatoma cells which show normal induction of most immediate-early genes. Sequence analysis revealed that *PRL-1* encodes a 19 kD novel protein that contains the 8 amino acid consensus protein tyrosine phosphatase (PTPase) active site. PRL-1 has no homology to other PTPases outside this domain leading us to conclude that it is a member of a new class of PTPases. Bacterially expressed PRL-1 is able to dephosphorylate the phosphotyrosine analog, p-nitrophenyl phosphate (PNPP), but shows no activity for serine-threonine phosphorylated substrates. PRL-1 itself contains consensus sites for tyrosine phosphorylation, is phosphorylated in vitro by Src kinase, and shows some ability to autodephosphorylate. Antibody localization studies indicate that PRL-1 is present in both cytoplasmic and nuclear fractions of cells. Among immediate-early genes, PRL-1 is the first PTPase to be identified, and like the cdc25 family of nuclear PTPases, may have an important role in cell cycle regulation in the mammalian growth response.

BZ 635 PROTEIN TYROSINE PHOSPHATASE α (PTPα) IS PHOSPHORYLATED BY PROTEIN KINASE C ON TWO SERINES CLOSE TO THE INNER FACE OF THE PLASMA MEMBRANE, Sharon Tracy, Peter van der Geer, and Tony Hunter, Molecular Biology and Virology Laboratory, The Salk Institute, PO Box 85800, San Diego, CA 920186, USA

PTPα,which has alternatively been referred to as HPTPα (Kruegger et al.), LRP(Mathews et al.), and R-PTP-α (Sap and Kaplan), is a member of the newly described family of protein tyrosine phosphatases (PTPases). PTPα is constitutively phosphorylated in NIH3T3 cells, predominantly on two serine residues and to a lesser extent on threonine and tyrosine. Phosphorylation of these serines is rapidly increased after TPA stimulation of NIH3T3 cells. Bacterially expressed PTPα is phosphorylated at these same sites by purified PKC. A combination of phosphotryptic mapping and site directed mutagenesis has identified these sites as serine 180 and serine 204 which lie close to the plasma mebrane in a region preceeding the catalytic domains. Although there are other minor unidentified sites of phosphorylation in PTPα, our data demonstrate that most of the phosphate content of PTPα can be directly attributed to PKC. These serine residues have been mutated to alanine. The wild type and mutant PTPαs are being expressed in bacteria to assess any changes in *in vitro* PTPase activity following PKC phosphorylation. Mutant PTPαs are also being expressed in NIH3T3 cells to determine the role of phosphorylation of PTPα in the response to the activity of ligand-activated receptor tyrosine kinases such as the PDGF receptor.

BZ 636 REGULATION OF TYPE-1 PROTEIN PHOSPHATASE BY SERINE/THREONINE AND TYROSINE PHOSPHORYLATION, Emma Villa-Moruzzi, Department of Biomedicine, University of Pisa, 56126, Pisa, Italy

Protein phosphatase of type-1 (PP1) is the most abundant serine/threonine phosphatase in most cells and is regulated by hormones and growth factors and during cell cycle. PP1 catalytic subunit exists in various active (E_a) and inactive (E_i) conformations and is purified either as free catalytic subunit or bound to the regulatory subunits inhibitor 2 (I2) and G. Regulation of PP1 is primarily through phosphorylation of its regulatory subunits: I2 phosphorylation by the kinase F_A/GSK3 and Casein Kinase II activate the cytosolic PP1; an insulin-stimulated kinase activates PP1 by phosphorylating the G-subunit in muscle glycogen particles, and also protein kinase A inhibits PP1 through the G-subunit. We have found that cdc2, the kinase that is activated at both mitotic and meiotic M-phase in eukaryotic cells, activates inactive PP1 (E_i•I2 complex, purified from skeletal muscle). Likewise the case of PP1 activation by F_A/GSK3, activation by cdc2 is accompanied by phosphorylation of I2 and free I2 can be phosphorylated as well. Correlation between PP1 activation and I2 phosphorylation is suggested by the fact that both activation and phosphorylation 1) increase in parallel during incubation with cdc2, 2) decrease in parallel upon subsequent cdc2 inhibition by EDTA, 3) are inhibited by the cdc2 inhibitor 5,6-dichlorobenzimidazole riboside. cdc2 phosphorylates also the catalytic subunit of PP1, whether in the complex with I2 or as free molecule. PP1 may be also regulated by direct phosphorylation of the catalytic subunit by tyrosine kinases. Some years ago it was reported that PP1 catalytic subunit was phosphorylated and inactivated by the kinases c-src and v-src. We have found that the catalytic subunit of PP1 is phosphorylated also by the tyrosine kinase v-abl as follows: 1) cytosolic PP1 is phosphorylated more (0.73 mol/mol) than PP1 obtained from the glycogen particles (0.076 mol/mol), while free catalytic subunit isolated in active or inactive form from cytosolic PP1 complex is phosphorylated even less and catalytic subunit complexed with I2 is not phosphorylated; 2) phosphorylation stoichiometry is dependent on the concentration of PP1 and 3 h incubation at 30° is required for maximal phosphorylation; 3) phosphorylation is on a tyrosine residue located in the C-terminal region of PP1 which is lost during proteolysis; 4) phosphorylation does not affect enzyme activity but allows conversion from active to inactive form upon incubation with I2 of a PP1 form that in its dephospho-form does not convert.

BZ 637 SPECIFIC BINDING OF THE SH2 DOMAINS OF HCP TO TYROSINE PHOSPHORYLATED PROTEINS IN HEMATOPOIETIC CELLS, Taolin Yi and James N. Ihle, Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN, 38105.

Hematopoietic Cell Phosphatase (HCP) is a novel protein tyrosine phosphatase predominantly expressed in hematopoietic tissues and cells (Yi, et al., Blood, 1991 and Yi, et al., Mol. Cell. Biol., 1992). It has also been identified and cloned from epithelia carcinoma cell lines under the name of PTP1C, SHPTP1 and the function of HCP in these cells is not clear. HCP protein tyrosine phosphatase has a single catalytic domain at the C terminal region and, interestingly, two SH2 domains at its N terminal region. To study the function of HCP in hematopoietic cells, HCP and its different domains were expressed and prepared as fusion proteins to Glutathione-S-Transferase (GST) in E. coli. The binding of these fusion proteins to tyrosine-phosphorylated substrates in hematopoietic cells induced by growth factors and PTK oncogenes were examined. We found that the SH2 domains of HCP preferentially bind to a panel of p-tyr proteins in different cells. While each of the two SH2 domains could mediate binding, and they appeared to recognize different phospho-tyrosine residues, the binding was most efficient in the presence of both SH2 domains. The binding between the SH2 domains and cellular proteins required the tyrosine phosphorylation of cellular proteins but not the SH2 fusion proteins. Among the known p-tyr proteins in hematopoietic cells, the SH2 domains of HCP did not bind to c-src or v-src PTK, although it bound to some of the v-src-phosphorylated substrates. Whereas the SH2 domains did not bind to phosphorylated c-fms PTK (and v-fms) in BAC1.2F5 stimulated with CSF-1, they bound to c-kit PTK and the binding was dependent on the activation and phosphorylation of c-kit kinase in response to SCF (MGF) stimulation. The specific binding of HCP SH2 domains to c-kit kinase indicates the potential involvement of HCP in signal transduction of SCF (MGF).

Phosphorylation/Dephosphorylation in Human Disease

BZ 638 AN EVOLUTION OF REARRANGEMENTS IN AMPLIFIED EGFR GENES IN HUMAN GLIOBLASTOMAS IN VIVO. V. Peter Collins, A. Jonas Ekstrand, Noriaki Sugawa, and C. David James. Ludwig Institute for Cancer Research, Clinical Group, Box 60004, S-10401 Stockholm, Division of Neuropathology, Dept. of Pathology I, Sahlgrenska Hospital, Gothenburg, Sweden and Dept. of Paediatrics, Division of Haematology/ Oncology, Emory University School of Medicine, Atlanta, Georgia.

Amplified EGFR genes in glioblastomas have been shown to be rearranged in the 5' region, resulting in aberrant, identically spliced transcripts lacking codons for the 6th through 272nd amino acids of the receptor's extracellular domain. Here we describe 8 cases of glioblastoma, with amplified EGFR genes displaying 3' internal deletion-rearrangements which over-express transcripts that lack sequences for a portion of the cytoplasmic domain believed to be essential for receptor down-regulation and thus attenuation. Significantly, three of the tumours displayed both 5' and 3' alterations, and in one of these, the alterations were acquired as separate events, the 5' mutation occurring between primary operation and recurrence one year later. In one case the 3' rearrangement results in the aberrant splicing of an exon to a cryptic splice site in an exon and the loss of 85 codons in the transcript which remains in frame. In the other cases the aberrant splicing of non-consecutive exons results in a frame shift with a stop codon and the transcript will code for a receptor with an intact tyrosine kinase region but with a truncated C-terminal end. In vitro experiments with artificially altered EGFR genes have established that each of these alterations should present the affected cell with a growth advantage. In combination with these results, the data presented here indicate an evolution of rearrangements in amplified EGFR genes in glioblastomas.

BZ 639 PHOSPHORYLATION AND BIOCHEMICAL ANALYSIS OF NM23. A. De La Rosa, N. J. MacDonald, M. A. Benedict, J. M. Perez Freije, and P. S. Steeg. Laboratory of Pathology, National Cancer Institute, Bethesda MD 20892.

The *nm23* cDNA was originally identified in murine melanoma cells on the basis of its reduced expression in high metastatic cell lines. Two human *nm23* cDNA clones, *nm23*-H1 and *nm23*-H2, have been reported. Transfection of the murine *nm23* gene into mouse melanoma cells has been shown to significantly suppress tumor metastatic potential *in vivo* and responsiveness to cytokine signals *in vitro*. The biochemical mechanisms of *nm23* suppressive activity is under investigation. The *nm23* gene product has been shown to be a nucleoside diphosphate kinase (NDP kinase), a class of ubiquitous enzymes which catalyze the transfer of the terminal phosphate of nucleoside triphosphates to nucleoside diphosphates. The phosphate transfer occurs through a ping-pong mechanism of action, via a high-energy phospho-histidine intermediate. Incubation of purified NM23 with ^{32}P-γATP or MCF-7 cells with ^{32}P-orthophosphate resulted in ^{32}P-phosphoprotein formation. In the *in vitro* reactions, NM23-H1 protein was autophosphorylated to a greater extent than NM23-H2. Biochemical analyses have shown that the phosphorylated amino acid is a serine. Site directed mutagenesis at the proposed NDP kinase phosphate transfer site, and thermodynamic considerations, indicate that the bond energy on the phosphoserine is insufficient for subsequent transfer of the phosphate to a nucleoside diphosphate. These data establish NM23-phosphoserine formation as distinct and independent from its NDP kinase activity. The functional significance of this serine phosphorylation will be tested in transfection experiments.

BZ 640 DIFFERENTIAL REGULATION OF AMPHIREGULIN AND TGF α EXPRESSION IN HUMAN SKIN. James T. Elder, Long-qing Xia, and Bhushan D. Hardas, Department of Dermatology, University of Michigan, Ann Arbor, MI 48109-0672

Amphiregulin (AR) belongs to the EGF family of growth factors, as evidenced by similarities to EGF and transforming growth factor-α (TGF-α) in EGF receptor binding, gene structure, and amino acid sequence. Recently, AR has been identified as an autocrine growth stimulator for normal human keratinocytes (KC). Unlike EGF and TGF-α, however, AR binds to and is antagonized by heparin, suggesting that AR may interact with ECM proteoglycans to regulate KC growth. In order to compare the regulation of AR and TGF-α in KC and in human skin, a 390 bp PCR product was amplified by reverse transcription and PCR using the primers TCT GGG GAC CAC AGT GCT GAT (forward) and GCA TGT TAC TGC TTC CAG GTG (reverse). The 390 bp PCR product was cloned by blunt-end ligation into pBSK$^+$ and sequenced, and a clone yielding the expected AR sequence was used to detect AR transcripts on Northern blots. AR transcripts were nearly undetectable in normal epidermal keratome biopsies, but were markedly increased (about 10-fold) in psoriatic lesions, as previously observed for TGF-α (Science 243:811, 1989). In contrast to TGF-α, however, AR transcripts were markedly increased during short-term organ culture of normal or psoriatic skin biopsies in defined medium containing no serum or polypeptide growth factors. These culture conditions are also known to couse rapid increases in the expression of c-myc, c-fos, and c-jun protooncogenes, whereas TGF-α is unaffected (JID 94:19,1990). Treatment of quiescent cultured KC with TGF-α (4-8 hr, 20 ng/ml) resulted in EGF receptor (EGFR) tyrosine kinase activation and increased AR mRNA levels by two-fold. Actinomycin D experiements revealed a marked increase (from 2 hr to >8 hr, n=3) in the half-life of AR transcripts after TGF-α treatment. Activation of protein kinase C with the phorbol ester TPA markedly increased TGF-α mRNA levels, but had little effect on AR steady-state mRNA levels or mRNA half-life in quiescent KC. Our results indicate that TGF-α is not the only EGF-like growth factor overexpressed in psoriatic lesions, and demonstrate that two such autocrine factors (TGF-α and AR) display different modes of regulation in the cutaneous environment. Finally, increased mRNA stability appears to be an important mechanism by which AR mRNA levels are regulated in response to EGFR activation in KC.

BZ 641 **EXPRESSION OF BCR/ABL IN MULTI-POTENT STEM CELLS CAN INITIATE DEREGULATED GROWTH, BUT IS NOT SUFFICIENT TO INDUCE LEUKEMOGENESIS.** Mikhail L. Gishizky, James Johnson-White and Owen N. Witte. Department of Microbiology and Medical Genetics, Howard Hughes Medical Institute, University of California - Los Angeles, Los Angeles, CA 90024.

BCR/ABL can affect the growth of murine multipotent stem cells in vivo and in vitro. Lethally irradiated mice reconstituted with bone marrow containing BCR/ABL expressing multipotent stem cells can develop a late onset (>20 wk) granulocytic leukemia that resembles the initial phase of human CML. In contrast to previous studies that described an early onset granulocytic hyperplasia, the late onset granulocytic leukemia can be propagated by adoptive transfer into sublethally irradiated syngeneic recipients. The transplanted mice develop a range of hematopoietic disorders that originate from the same stem cell, these include chronic granulocytosis, acute myeloid, B- and T-lymphoid leukemias. In vitro, BCR/ABL can initiate the deregulated growth of multipotent stem cells without blocking their ability to differentiate. Myeloid and lymphoid cell lines derived from the same BCR/ABL expressing stem cell initially require the presence of specific cytokines for growth. With time in culture, the cells become growth factor independent. Interestingly, the factor independent cell lines are non tumorigenic, indicating that additional somatic changes are required for development of a malignant phenotype. Retroviral transduction of secondary oncogenic events into the growth factor independent cell lines can lead to malignant transformation. These studies demonstrate that BCR/ABL induced transformation, which originates from multipotent stem cells, progresses through discrete stages that require the acquisition of additional genetic events. Using these two model systems we can begin to identify the specific events responsible for this progression.

BZ 642 EPIDERMAL EXPRESSION OF KGF CAUSES REMARKABLE CHANGES IN THE SKIN OF TRANSGENIC MICE, Lifei Guo, Qian-Chun Yu, and Elaine Fuchs, Howard Hughes Medical Institute, Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637

Keratinocyte growth factor (KGF) belongs to the seven member fibroblast growth factor family. Synthesized by cells of the dermal component of skin, KGF's potent mitogenic activity is on the epidermal component. KGF differs from TGFα in that it is a paracrine, rather than autocrine, growth factor for epidermal keratinocytes. In addition, KGF is considerably more mitogenic to keratinocytes than TGFα. To explore the possible role of KGF in mesenchymal-epithelial interactions in skin and to ascertain how the effects of KGF and TGFα on epidermal growth and differentiation may differ, we used a human keratin 14 promoter to target expression of human KGF cDNA to the stratified squamous epithelia of transgenic mice. Mice expressing KGF in their epidermis showed dramatic skin abnormalities. While some of these changes resembled those seen when TGFα was overexpressed in skin with the same K14 promoter, many effects were strikingly different. In addition, the skin displayed several remarkable and unexpected changes in hair follicle morphogenesis and adipogenesis, which were not seen in TGFα mice. Interestingly, some of these effects were seen in animals that expressed KGF at levels that were too low to cause epidermal abnormalities. Collectively, our findings represent the first *in vivo* study on KGF and suggest that KGF is a potent growth factor which can elicit global effects on the growth and differentiation of many cells types within the skin. Furthermore, the major differences between K14-KGF and K14-TGFα mice imply that these growth factors must mediate their effects on epidermal keratinocytes through different pathways.

BZ 643 UNCOUPLING OF PURINERGIC RECEPTORS FROM PHOSPHOLIPASE C IS ASSOCIATED WITH LOSS OF GROWTH REGULATION IN A PROSTATE CARCINOMA CELL LINE, M.J. Ha[1], W.K. Kang[1], Y.-J. Bang[1], R.T. Jensen[2], W.-G. Fang[1], A. Shenker[3], A.M. Spiegel[3], D.J. Park[4], M. L. Koenig[5], F. Pirnia[1], C.E. Myers[1], P.K. Goldsmith[3], W.T. Niklinski[1] and J.B. Trepel[1], [1]Clinical Pharmacology Branch, National Cancer Institute, [2]Digestive Diseases Branch and [3]Molecular Pathophysiology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, [4]Laboratory of Biochemistry, National Heart Lung and Blood Institute, National Institutes of Health, Bethesda, MD, 20892, and [5]Department of Medical Neurosciences, Walter Reed Army Institute of Research, Washington, D.C. 20307.

To find a new therapeutic approach to advanced metastatic prostate cancer we tested human prostate carcinoma cell lines derived from metastatic sites for expression of plasma membrane receptors that transduce a growth-inhibitory signal. In 3/3 androgen-independent lines P_2-purinergic receptor agonists induced activation of phospholipase C (PLC), a dramatic increase in cytoplasmic free Ca^{2+}, prolonged oscillations in cytoplasmic and nuclear free Ca^{2+}, and growth arrest. In contrast, in the human androgen-sensitive cell line LNCaP, P_2 agonists had no effect on PLC activity, Ca^{2+} mobilization, or cell growth. Binding studies showed the K_d and B_{max} for the P_2 receptor of LNCaP cells was indistinguishable from the responder cell line PC-3-M. Thus LNCaP cells express P_2 receptors uncoupled from PLC. All prostate carcinoma cell lines showed expression of PLC-β1 by Western blot analysis, suggesting that the defect in post-receptor signalling may be at the level of the heterotrimeric G protein. While all lines showed similar expression of β subunits, there was a marked difference between LNCaP and the responder lines in α subunit expression. The 3 responder lines had abundant plasma membrane expression of most of the Gα subunits including α_s, α_{i2}, α_{i3}, $\alpha_{q/11}$, α_{13}, and α_z. LNCaP membranes had low to undetectable expression of α subunits except α_s and α_{i3}.These data demonstrate that uncoupling of P_2 receptors from PLC is associated with loss of growth regulation through that receptor in a prostate carcinoma cell line. Furthermore, these data suggest that the lesion in post-receptor signalling may be at the level of G protein plasma membrane α subunit expression.

BZ 644 A RETROVIRUS GENE-TRAP STRATEGY FOR TARGETING AND ISOLATING GENES WHICH NEGATIVELY REGULATE TRANSFORMATION.

Geoffrey G. Hicks and H. Earl Ruley, Department of Microbiology and Immunology, Vanderbilt University, Nashville, TN, 37232.

The rat embryo fibroblast cell line REF52 can be efficiently transformed by cotransfection of an activated *ras* and either SV40 large T antigen or Adenovirus E1A genes. Unlike other fibroblast model systems however, expression of *ras* alone does not transform but leads to cell cycle arrest at either G2/M or G1/S, gross morphological changes, and subsequent cell death. Factors responsible for *ras*-induced growth/transformation suppression can be transfered by somatic cell hybridization in a dominant manner. Further studies have suggested that known tumor suppressor genes like Rb or p53 are not responsible for this activity. The REF52 cell line therefore, provides a very tight genetic system to study novel factors that regulate cellular responses to *ras* oncogenes. We have developed a retroviral gene-trap strategy to identify the gene(s) responsible for this activity by insertional mutagenesis. The retroviral construct, U3neoCMVras, contains a *neo* gene in the U3 region of an enhancerless LTR, hence neomycin resistance is confered only when the virus integrates into expressed cellular genes. p21 $ras^{T24/EJ}$ is concomitantly expressed from a CMV promoter within the body of the provirus. Expression of *neo*^{r} and high levels of p21 $ras^{T24/EJ}$ in transformed cell lines is expected to arise by integration events that disrupt the putative growth/transformation suppressor gene, followed by loss of the unoccupied allele. Cells which have lost a gene required for *ras* function may also emerge as untransformed clones expressing high levels of p21 $ras^{T24/EJ}$. A gene-trap library of 40,000 neo^r colonies (sufficient to disrupt every actively expressed gene) has been isolated. The library is being screened for the ability of individual colonies to maintain themselves in continuous culture, for expression of transformed phenotypes, and for the ability to form tumors in nude mice. Potential genes that regulate cellular responses to *ras* will be isolated from sequences near the sites of virus integration.

BZ 645 PRIMARY T CELL IMMUNODEFICIENCY ASSOCIATED WITH ABNORMAL TYROSINE PHOSPHORYLATION OF CELLULAR PROTEINS FOLLOWING T CELL RECEPTOR/CD3 CROSSLINKING

Claire Hivroz, Françoise Le Deist, Hélène Buc, and Alain Fischer, INSERM U132 Hopital Necker, Paris, France.

A T cell immunodeficiency (ID) was detected in a 13 year-old girl with repeated opportunistic infections, protracted diarrhea and autoimmune hemolytic anemia whose sister died from infections. The ID is characterized by a T lymphocyte activation deficiency. T-cell activation-induced by antigens, allogeneic cells, PHA, anti-CD3 and anti-CD2 antibodies was defective as measured by proliferation, IL-2 receptor expression, interferon-γ production and phosphoinositide turn over, while Ca^{2+} flux was found normal. No membrane markers abnormality could be found on patient's cells. Defective T cell activation was localized at a very early step of activation by studying phosphorylation on tyrosine following anti-CD3 stimulation. Western blot analysis with anti-phosphotyrosine antibodies revealed a reduced number of phosphorylated proteins since 5 bands of apparent molecular weight 170, 135, 100, 75 and 46 kDa were missing. Phosphorylation of PLC-γ1 on tyrosine residues in response to TCR/CD3 triggering could not be detected in the patient's cells. Activities of $p56^{lck}$ and $p59^{fyn}$, two of the major tyrosine kinases associated with the CD4/CD8/CD3/TCR complex, were found normal as measured by their autophosphorylation. We thus describe a primary T cell ID characterized by an early T cell activation defect caused either by an undetected tyrosine kinase primary anomaly or by a defective regulatory element of tyrosine kinase activity.

BZ 646 ENHANCEMENT OF PLC ACTIVITIES BY TYROSINE PHOSPHORYLATION IN HUMAN COLORECTAL CARCINOMAS.

Miwako K. Homma, Yoshimi Homma[1], Masayoshi Namba[2], Tadaomi Takenawa[3], Yasuhito Yuasa. Department of Hygiene &Oncology, Tokyo Medical & Dental University, 1-5-45, Yushima, Bunkyo, Tokyo 113, [1]Tokyo Metropolitan Inst. of Gerontology, [2]Okayama University, [3]University of Tokyo, Japan

The production of the second messenger molecules diacylglycerol and inositol 1,4,5-trisphosphate is mediated by activated phosphatidylinositol-specific phospholipase C (PLC) enzymes. We report the enhancement of PLC activity in human colorectal carcinoma cells. PLC activity *in vitro* was increased in cell lines derived from human colorectal carcinomas, especially in KMS-4 and KMS-8 cells, both of which are derived from FAP (Familial Adenomatous polyposis) patients. In these cells, cellular contents of inositol 1,4,5-trisphosphate and diacylglycerol were constitutively increased. When cell lysates were phosphorylated and immunoprecipitated with anti-phosphotyrosine antibody, phosphorylated PLC-γ_1 was detected as a 145 KDa band; this phosphoprotein was not detected in lysates from normal colorectal cells. In addition, activation of membrane-bound protein kinase C was also observed in these carcinoma cells. These results suggest the involvement of phospholipase C activation induced by its tyrosine phosphorylation, followed by enhanced metabolism of inositol phospholipids and consequent activation of protein kinase C in human colorectal carcinogenesis.

BZ 647 CO-AMPLIFICATION AND OVEREXPRESSION OF THE GENES FOR PDGF-α-RECEPTOR AND c-kit IN A HUMAN GLIOBLASTOMA.

C. David James[1], V. Peter Collins[2,3] and A. Jonas Ekstrand[1,2]. [1]Dept. Neurosurgery, Emory University School of Medicine, Atlanta, Georgia 30322, U.S.A., [2]Ludwig Institute for Cancer Research, S-104 01 Stockholm, Sweden. [3]Div. Neuropathology, Dept. Pathology, Sahlgrenska Hospital, Göteborg, Sweden.

We describe in a human glioblastoma biopsy the co-amplification and overexpression of two genes; platelet-derived growth factor-α-receptor (PDGFRA) and c-kit (KIT). Each of these genes codes for subclass III transmembrane growth factor receptors with a split protein tyrosine kinase domain. No structural abnormalities of the amplified PDGFRA and KIT genes, nor of their corresponding mRNAs could be detected using Southern, northern and PCR analysis. No amplification of the genes for EGFR, c-myc, N-myc or gli was detected in this specimen, as have been identified in other gliomas and/or their cell lines.

Both cytogenetic and molecular genetic analyses have established a close physical linkage of PDGFRA and KIT on chromosome 4, and it is possible that their co-amplification is a consequence of their proximity. Since the other components of the PDGF-sytem, namely the PDGF A- and B-chains as well as PDGF-β-receptor are expressed in glioma tissue and cell lines, it is reasonable to speculate that PDGFRA is the target of this genetic alteration and that overexpression of PDGF-α-receptor activates the PDGF growth stimulatory loop. Conversely, the overexpression and/or interaction of both receptor tyrosine kinases may be necessary to promote growth in the affected cells.

BZ 648 *DELETION OF THE p58 PROTEIN KINASE GENE IN HUMAN* NEUROBLASTOMA, Jill M. Lahti[1], Marcus Valentine[2], Gail Richmond[1], Jose Grenet[1], Bart Jones[2], A. Thomas Look[2], and Vincent J. Kidd[1], Departments of [1]Tumor Cell Biology and [2]Hematology/Oncology, St. Jude Children's Research Hospital, Memphis, TN 38105.

The process of cell division control in mammals is complex, and appears to require a number of proteins. Among these, the $p34^{cdc2}$-related protein kinases and the cyclins may represent the largest, most reiterated gene families. *The functions of these reiterated cell cycle* gene products has not clearly been established, but recent evidence suggests that many, if not all, of these proteins may contribute to normal cell cycle regulation. We have previously described the isolation and characterization of one of the $p34^{cdc2}$-related genes, p58. This protein kinase is ubiquitously expressed and evolutionarily conserved. Minimal overexpression of this protein kinase in eukaryotic cells results in cell cycle delay in late telophase, increased doubling time, and substantially increased frequencies of mulitnucleated and micronucleated cells. Conversely, dimunition of p58 protein kinase activity leads to decreased cell doubling time and increased cell proliferation. The p58 gene localizes to human chromosome 1p36. This chromosome region is deleted in many tumors during the late stages of disease. Loss of the 1p36 region coincides with increased growth capacity of many of these tumors. We have examined one of the tumor types, neuroblastoma, to determine whether alterations in p58 may be involved in tumorigenesis. Of 18 human neuroblastoma cell lines with 1p abnormalities studied, we found that the p58 protein kinase gene was deleted in 14/18 and translocated to another human chromosome in 4/18. In one of the cell lines with a p58 deletion, the remaining p58 gene has been altered by an insertion of approximately 2.5 kb in the promoter region. Five of six cell lines with p58 deletions have substantially reduced p58 mRNA (< 50% normal). Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of the remaining expressed p58 mRNA from two neuroblastoma cell lines indicates that this mRNA is normal, even though it is reduced in abundance. We are now examining p58 protein levels and protein kinase activity in all of these cell lines. These studies may provide some insight into the possible correlation between decreased p58 expression and activity and the observed changes in the growth capacity of these tumors following the loss of the p35-36 region of chromosome 1, thereby linking cell cycle protein kinase genes and tumorigenesis.

BZ 649 **THE *E5* ORF OF HUMAN PAPILLOMAVIRUS TYPE 16 TRANSFORMS CELLS AND PERTURBS THE DOWN-REGULATION AND ACTIVITY OF THE EPIDERMAL GROWTH FACTOR RECEPTOR,** Dennis J. McCance, Samuel S. Straight, Patricia M. Hinkle*. Department of Microbiology and Immunolgy, Pharmacology*, University of Rochester, NY 14642.

The E5 open reading frame (ORF) of the human papillomaviruses (HPV) encodes a short hydrophobic peptide, which can transform rodent fibroblasts and extent the life span of primary human keratinocytes. The rate of transformation of rodent fibroblasts is increased in the presence of EGF, but not PDGF. E5 acts as a mitogen in resting keratinocytes and when EGF was added concurrently, there was a synergistic response. Further investigation of the role the EGF receptor (EGFR) showed that in E5 expressing keratinocytes there was (i) an increase in the number of receptors by 2- to 4- fold, (ii) no change in the rate of internalization of the receptor, but the degradation of the EGF/EGFR complex in endosomes was delayed, (iii) an increase in the phosphorylation of the receptor in E5 containing cells after ligand stimulation, but the constitutive activity was the same as control cells. The delay in degradation appeared to result in the recycling of approximately 40% of receptors as opposed to the recycling of 7% in control cells. The availability of receptors without the necessity of *de novo* synthesis may explain the activity of E5 in keratinocytes.

BZ 650 THE EFFECT OF EPSTEIN-BARR VIRUS LATENT MEMBRANE PROTEINS 2A AND 2B (LMP2A/LMP2B) ON CELL SURFACE SIGNALLING IN B LYMPHOCYTES, [1]Cheryl L. Miller, [2]Richard Longnecker, and [2]Elliott Kieff, [1]Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago Il 60637, [2]Departments of Medicine and Microbiology and Molecular Genetics, Harvard Medical School, 75 Francis Street, Boston, MA 02115

Epstein-Barr Virus (EBV) is an important human pathogen which is the causative agent of infectious mononucleosis, and is etiologically linked with B lymphocyte and epithelial cell malignancies. In vitro, the EBV host range is restricted to B lymphocytes which are largely nonpermissive for virus replication. Latently EBV infected lymphocytes are driven to perpetually proliferate and are tumorigenic in SCID mice, marmosets, and immune compromised humans. EBV encodes three integral membrane proteins in latently infected growth-transformed cells. One of these, LMP1, induces the expression of lymphocyte activation associated molecules, and is capable of transforming rodent fibroblasts. The remaining two EBV-encoded membrane proteins are LMP2A and LMP2B. These proteins are expressed from alternatively spliced transcripts of the LMP2 gene which differ only in their first exon usage. The LMP2A first exon contains an initiator of translation which results in an amino terminal hydrophilic sequence which LMP2B lacks. LMP2 proteins colocalize with LMP1 in a constitutive patch in EBV-transformed B lymphocyte plasma membranes. LMP2A interacts with, and acts as a substrate for, members of the *src* tyrosine kinase family. Particular tyrosine kinases with which LMP2A has been shown to associate with are implicated in immunoglobulin-mediated signal transduction.

The association of LMP2 proteins with LMP1, and with members of the *src* tyrosine kinase family, suggests that LMP2 proteins interact with B cell signal transduction pathways. Our studies indicate that the stable expression of LMP2A in a B-cell line interferes with the Ca^{2+} mobilization that normally occurs upon the crosslinking of surface IgM, class II, or CD19 molecules. Interestingly, expression of LMP2A also results in the reduced expression of surface IgM. We are currently defining which LMP2 domains are responsible for these effects.

BZ 651 EFFECTS OF KINASE INHIBITORS/ACTIVATORS ON UPTAKE OF *CITROBACTER FREUNDII* AND *SALMONELLA TYPHI* INTO HUMAN EPITHELIAL CELL LINES, Tobias A. Oelschlaeger and Dennis J. Kopecko, Department of Bacterial Immunology, WRAIR, Washington, DC 20307-5100

Recently, we have shown that epithelial cell internalization of *Citrobacter freundii*, strains 3009 and 3056, is uniquely dependent on microtubules and coated pit formation. This contrasts with the microfilament-dependent invasion displayed by many bacterial pathogens, of which *Salmonella typhi* Ty2 was chosen as a representative example. To further characterize these different uptake mechanisms, invasion assays were performed with a variety of cell lines in the presence or absence of different kinase inhibitors/activators. The inhibitor/activator was added 1 hr prior to addition of bacteria to the epithelial cell monolayer and was maintained during the 2hrs invasion period. Then the monolayer was washed and incubated for another 2 hrs with medium containing gentamycin to kill extracellular bacteria. Finally, the epithelial cells were lysed with 0.1% Triton X-100 and released bacteria were enumerated by plate count. Preliminary results have shown that Ty2 uptake in all cell lines was dramatically reduced only by H-89, an inhibitor of cAMP-dependent kinase. In contrast, invasion by *Citrobacter* 3009 and 3056, not as markedly reduced by H-89, severely decreased in the presence of genistein, a tyrosine-specific kinase inhibitor. Activation of protein kinase C by phorbol 12,13-dibutyrate had no effect on entry of Ty2, 3009 or 3056 into cultured human epithelial cells.

BZ 652 CHARACTERIZATION AND PURIFICATION OF THE HUMAN RECEPTOR FOR THE HEAT-STABLE ENTEROTOXIN, Vasanthi Ramachandran, S. Ramamohan & Sandhya S. Visweswariah, Astra Research Centre India, P.B. No. 359, 18th Cross Malleswaram, Bangalore 560 003, India.

The heat stable enterotoxin (ST) of *Escherichia coli*, one of the causative agents for acute diarrhoea is a specific activator of membrane bound guanylyl cyclase which causes an accumulation of intracellular cGMP followed by chloride secretion by intestinal cells. ST receptor in the human colonic carcinoma cell line (T84) was identified and characterized using a novel iodinated mutant ST peptide ($^{125}IST_{Y72F}$) where the tyrosine at the 19th position in the STh peptide was changed to phenylalanine by site directed mutagenesis. Scatchard analysis of the binding data indicated that the ST receptor had an affinity of ~ 10^{-10} M. Binding of ST to its receptor was pH dependent with little change in affinity at pH 5.0 as compared to pH 7.5, but with an increase in capacity at lower pH. This increase in ST binding at pH 5.0 was reflected in a corresponding increase in cGMP production. This property gains physiological importance when one considers the acidic micro-environment of the small intestinal mucosa. Covalent crosslinking of $^{125}IST_{Y72F}$ to its solubilized receptor indicated that the molecular weight of the receptor was 160,000 Da. Purification of the ST receptor was accomplished using GTP agarose and wheat germ lectin agarose affinity chromatography. A major band of molecular weight 160,000 Da was detected in denaturing gels following silver staining. The purified receptor could bind $^{125}IST_{Y72F}$ with high affinity and specificity, and possessed guanylyl cyclase activity. A 90% reduction in the levels of cGMP produced was observed on prolonged exposure of the T84 cells to ST. This phenomenon was specific since cholera toxin did not bring about a similar effect. The above observation indicated that there was specific regulation of expression of the ST receptor.

BZ 653 THE PAPILLOMAVIRUS E5 ONCOPROTEIN: TRANSFORMING ACTIVITY AND BINDING TO THE CELLULAR TARGETS, 16K AND PDGF RECEPTOR, ARE STRINGENTLY REGULATED BY ITS TRANSMEMBRANE GLUTAMINE RESIDUE. Jason Sparkowski, Thorkell Andresson, David Goldstein and Richard Schlegel. Department of Pathology, Georgetown University, Washington, DC 20007.

The major transforming protein of bovine papillomavirus type 1 (BPV-1), E5, is a strongly hydrophobic 44 amino acid polypeptide. It mediates the tumorigenic conversion of immortalized rodent cells via a mechanism apparently involving transmembrane interactions with both the 16K membrane pore protein (a component of the vacuolar H^+-ATPase) as well as growth factor receptors, including the PDGF receptor. E5 contains two domains: a hydrophobic N-terminus responsible for membrane localization and a hydrophilic C-terminus containing two cysteine residues necessary for biological activity and homo-dimer formation.

A transmembrane glutamine residue, at position 17 within the hydrophobic domain of E5, is strongly conserved in the fibropapillomaviruses (BPV-1, BPV-2, DPV, EEPV and RPV) and limited mutagenesis of this residue has indicated that this amino acid is important for biological function and 16K binding. In the current study, we have performed extensive site specific mutagenesis at Gln17 and converted it into polar, non-polar, acidic, and basic residues in order to evaluate its potential role in E5 function. We have evaluated these mutant proteins for their transforming activity and binding to 16K and PDGF receptor. All mutant proteins were found to be biochemically stable and were expressed to similar levels in cos cells. The level of 16K binding by the E5 mutant proteins was observed to be variable and ranged from 20-120% of wild-type protein. Most interestingly, only one mutant protein, glutamine converted to glutamic acid, retained any transforming activity (approximately 20% of wild-type). All other mutants were entirely transformation defective despite their ability to bind varying levels of 16K. Preliminary data indicate that the binding properties of mutant E5 proteins to 16K are also reflected in the binding of PDGF receptor. Potential mechanisms of E5 transformation involving receptor-mediated signal transduction are discussed.

BZ 654 HUMAN HSB2 T LYMPHOMA CELLS CONTAIN AN ACTIVATED, MUTANT p56^{LCK}. Dwaine Wright#, Bart Sefton*, and Mark P. Kamps#. #University of California San Diego, School of Medicine, Department of Pathology, La Jolla, California 92093. *Salk Institute for Biological Studies, Molecular Biology and Virology Laboratory, 10010 North Torrey Pines Road, La Jolla, CA, 92037.

p56^{lck} is a membrane-associated tyrosine protein kinase of the src family that is expressed primarily in T cells. Oncogenic conversion via biochemical activation of src-family proteins can occur by deletion or specific mutation of their regulatory COOH-terminal tyrosine residue as well as by mutations in their SH2 and SH3 domains. Biochemical activation of these tyrosine protein kinases increases the level of autophosphorylation. In other instances, such as the LSTRA thymoma cell line, overexpression of normal p56^{lck} may also contribute to the leukemic phenotype.

We have used Western blotting with antibodies to phosphotyrosine and p56^{lck} to determine whether p56^{lck} is activated or overexpressed in any human T cell leukemias. One cell line, HSB2, was shown to exhibit a 14-fold increase in phosphotyrosine-containing proteins. It expresses a slightly larger form of p56^{lck} that is highly phosphorylated at its autophosphorylation site, implying that it is constitutively activated. p56^{lck} is not overexpressed in HSB2 cells. Sequence analysis of an HSB2 *lck* cDNA demonstrated that HSB2 Lck contains 3 point mutations and a 3 amino acid insertion at the COOH-terminal edge of the SH2 domain. We are currently determining whether these mutations are sufficient to convert p56^{lck} into an activated oncogene. Interestingly, HSB2 cells have been demonstrated to contain a t(1;7) translocation that inserts the T cell receptor enhancer between the proximal and distal LCK promoters, resulting in potential transcriptional deregulation. Because LCK transcription is downregulated coincident with T-cell activation, translocation with the T-cell receptor enhancer likely circumvents feedback inhibition of LCK transcription in the translocated allell in HSB2 cells, allowing persistant expression of mutant p56^{lck}.

Late Abstracts

TYROSINE PHOSPHORYLATION OF PLCγ AND FORMATION OF INOSITOL PHOSPHATES IS ASSOCIATED WITH THE ACTIVATION OF HUMAN PLATELETS BY PEROXOVANADATE Robert A. Blake, Trevor R. Walker, Steve P. Watson, Department of Pharmacology, Oxford University, U.K.

High levels of protein tyrosine phosphorylation can be induced in platelets by a peroxovanadate complex (Inazu *et al.*1990), which is formed in solutions of orthovanadate and hydrogen peroxide. We present evidence that peroxovanadate stimulates PLC activity through a kinase dependent pathway, possibly as a result of the tyrosine phosphorylation of PLCγ1; this may underlie the activation of platelets by peroxovanadate.

Protein Phosphorylation: The majority of tyrosine phosphorylated proteins were detectable within 20s of peroxovanadate addition (Sodium vanadate 400μM, H_2O_2 4mM). Staurosporine inhibited the tyrosine phosphorylation of all proteins except one of 54 kDa which co-migrated with *src*. Ro 31-8220 an analogue of staurosporine with increased selectivity for protein kinase C (Davis *et al* 1989, Walker and Watson (1992)) had no effect on tyrosine phosphorylation.

Functional responses: Aggregation, shape change and [^{3}H]5-HT secretion induced by peroxovanadate were inhibited completely by staurosporine (10μM). In contrast, Ro 31-8220 (10 μM) had no apparent effect on peroxovanadate-induced shape change but slowed the rate of aggregation and reduced markedly the degree of secretion.

Formation of [^{3}H]inositol phosphates: Peroxovanadate stimulated marked formation of total [^{3}H]inositol phosphates and this was inhibited completely in the presence of 10 μM staurosporine. In contrast, Ro 31-8220 (10 μM) caused a small increase in peroxovanadate-induced formation of total [^{3}H]inositol phosphates which did not reach statistical significance.

Tyrosine phosphorylation of PLCγ: Several tyrosine phosphorylated proteins were detectable in anti-PLCγ immunoprecipitates from control and stimulated platelets (anti-PLCγ generous gift from T. Pawson (Decker *et al.*). However, tyrosine phosphorylation of PLCγ1 was detectable only in immunoprecipitates from platelets treated with peroxovanadate (20s) despite the fact that immunoprecipitates from control and stimulated platelets contain similar levels of PLCγ1.

Davis, P.D.*et al.* (1989) FEBS Lett 259 61 - 63.
Decker, S.J *et al.*(1990) J.Biol.Chem. 265 7009 - 7015.
Inazu, T.*et al.* (1990) Biochem. Biophys. Res. Commun. 170 259 - 263.
Walker, T.R., & Watson, S.P. (1992) Biochem. J. *in press*

PREVENTION OF APOPTOSIS IN THE OLIGODENDROCYTE LINEAGE BY PROTEIN KINASE C DEPENDENT AND INDEPENDENT PATHWAYS. Huseyin Mehmet and Martin Raff, Biology Dept., Medawar Building, University College London, Gower Street, London WC1E 6BT, U.K.

The rat perinatal optic nerve has provided a useful model system for studying the development of oligodendrocytes, the major myelin-forming cells of the CNS. These cells develop on a precise schedule from a bipotential precursor, termed the oligodendrocyte-type-2 astrocyte (O-2A) progenitor cell since, *in vitro*, it can also give rise to type-2 astrocytes. The final number of oligodendrocytes in the optic nerve is not only dependent on O-2A progenitor cell proliferation and terminal differentiation, but is also determined by the survival of oligodendrocytes and their precursors. It has recently been shown that both these cell types depend on signals from other cells for their survival. These include platelet-derived growth factor (PDGF) and insulin-like growth factor-1 (IGF-1). If cells in culture are deprived of such survival factors, they will die, following the activation of an intrinsic death programme. What are the intracellular signalling events that promote the survival of O-2A lineage cells? In order to address this question, we have examined the effects of various second messenger agonists on cell survival. We found that phorbol-12,13-dibutyrate (PDBu), a direct activator of protein kinase C (PKC), is sufficient for the survival of O-2A progenitor cells. PDBu inhibits cell death in a dose-dependent manner, with a maximal effect at 10 nM. Furthermore, if cells are pretreated with a high concentration (200 nM) of this phorbol ester to down-regulate PKC, PDBu is no longer able to save O-2A progenitors. In contrast, IGF-1 promotes the survival of both control and PKC-downregulated cells. These results indicate that cell survival, like O-2A progenitor cell proliferation, can be stimulated by both PKC-dependent and independent pathways.

INHIBITION OF RETINOBLASTOMA PROTEIN PHOSPHORYLATION AND CELL CYCLE PROGRESSION BY THE PHOSPHATASE INHIBITOR OKADAIC ACID

Axel Schönthal* and James R. Feramisco+. +Departments of Medicine and Pharmacology, University of California, San Diego, La Jolla, CA 92093-0636; *Department of Microbiology, University of Southern California, 2011 Zonal Avenue, Los Angeles, CA 90033-1054.

Reversible phosphorylation events are major regulatory mechanisms of signal transduction pathways that regulate gene expression and cell growth. To study the potential involvement of serine/threonine specific phosphatases in these processes we used okadaic acid (OA), an inhibitor of type 1 and type 2A protein phosphatases.

Here we present evidence that OA arrests cells at defined points in the cell cycle. Concomitantly, expression and associated histone H1 kinase activity of cdc2 and cyclin A, two cell cycle regulatory proteins, are repressed by this agent. Furthermore, phosphorylation of the tumor suppressor protein retinoblastoma, an event thought to be necessary in order to permit cells to proliferate, is inhibited when OA is present. These effects are fully reversible since removal of OA restores cdc2 and cyclin A expression as well as histone H1 kinase activity, and the cells resume growth.

Since cdc2 and cyclin A have previously been shown to be absolutely required for cell cycle progression it is likely that blockage of synthesis of these components contributes to the cytostatic effects of OA. Furthermore, our results suggest a positive role for OA sensitive protein phosphatases in the regulation of expression of these cell cycle regulatory proteins.