Instituto Juan March
de Estudios e Investigaciones

106 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Control of Signalling by Protein Phosphorylation

Organized by

J. Schlessinger, G. Thomas, F. de Pablo and J. Moscat

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Introduction

J. Moscat and F. de Pablo

The phosphorylation and dephosphorylation of proteins and lipids are critical processes for the execution of the cellular programs leading to proliferation, differentiation and survival. The recent completion of the *D. melanogaster* genome and its comparison with that of other organisms such as *C. elegans and S. cerevisiae*, has revealed that protein kinases are among the top 10 protein families that are common to all three organisms and constitute aproximately 2% of each proteome. In Drosophila, the kinase group is constituted by aproximately 75 % serine/threonine kinases and 25% tyrosine or dual specificity kinases.The impressive recent progress in identifying kinases and phosphatases implicated in signal transduction in eukaryots has been paralleled by progress in defining their phosphorylation sites, the effect of phosphorylation on protein-protein interactions and consequences of their mutation or functional inactivation at the level of the cell an the whole organism.

This meeting has served as a lively forum for discussion of recent advances in the field of signal transduction and its regulation by protein phosphorylation in systems ranging from mammals to yeast. The first part of the workshop focused on the role of receptor tyrosine kinases, PI3K and the MAPK/ERK signaling pathways in cell growth, proliferation and survival. Another series of presentations discussed cytokine signaling with special emphasis in the mechanism of NF-κB activation, as a paradigm of cytoplasmic signaling transmitted from membrane to nucleus. The crystal structure of several ligand-receptor complexes, serine-threonine kinases and the tumor suppressor PTEN were presented. The last part of the workshop was devoted to developmental and differentiation models addressing issues such as cell size control, apoptosis in vivo and cell cycle regulation.

Jorge Moscat and Flora de Pablo

Session 1: Mechanisms of kinase activation

Chair: Jorge Moscat

From the fly to the mouse: the role of S6 kinase in cell growth

George Thomas

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We have been interested in the distinction between growth, or increase in cell mass, and proliferation increase in cell number. It is evident that a cell must first grow before it proliferates, otherwise it will divide into infinitely smaller cells, a process which could not go on indefinitely. Although we have learned a great deal concerning the mechanisms which control cell cycle progression, we know little concerning the molecular mechanisms which control cell growth. What is clear, is that they include all the anabolic processors required for a cell to duplicate itself. Chief amongst these is the activation protein synthesis. However what is largely overlooked is that the major products being synthesized are components of the translational machinery, most notably ribosomal proteins. The mRNAs encoding these proteins are characterized by a polypyrimidine at track of their 5' transcriptional start site. Earlier studies from our group have shown that the upregulation of these mRNAs is controlled at the translational level through S6 kinase, presumably mediated by increased 40S ribosomal protein S6 phosphorylation. Our present efforts have largely focussed on validating this model in an *in vivo* setting and establishing genetic screens to search for additional components in this signaling pathway. This presentation will review our recent studies in both *Drosophila* and the mouse in addressing this issue.

Physiological roles of PDK1 deduced from the embryonic stem cells lacking this protein kinase

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Protein kinase B (PKB, also called Akt) is activated within a minute or two when cells are stimulated with insulin or insulin-like growth factor-1 (IGF-1) and mediates many of the intracellular actions of these signals by phosphorylating key regulatory proteins at serine and threonine residues that lie in Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr sequences. Physiological substrates for PKB include glycogen synthase kinase-3 (GSK3), the PDE3B isoform of cyclic AMP phosphodiesterase, and the cardiac isoform of 6-phosphofructo-2-kinase (PFK2). The inhibition of GSK3 by PKB leads to the dephosphorylation and activation of glycogen synthase and protein synthesis initiation factor eIF2B and contributes to the insulin-induced stimulation of glycogen synthesis and protein synthesis. The activation of PDE3B underlies the insulin-induced decrease in the level of cyclic AMP, which inhibits lipolysis in adipose tissue. The activation of PFK2 underlies the insulin-induced stimulation of glycolysis in the heart.

The insulin or IGF1-induced activation of PKB results from the phosphorylation of PKB at two residues, namely Thr308 in the activation loop of the catalytic domain and Ser 473 near the C-terminus. The phosphorylation of both residues is prevented by inhibitors of phosphatidylinositol (PI) 3-kinase, indicating that PI(3,4,5)trisphosphate (PIP3), the product of the PI 3-kinase reaction, mediates the phosphorylation and activation of PKB. Nearly three years ago we identified and characterised a protein kinase that phosphorylates PKB at Thr308 provided that PIP3 is present in the reaction. We therefore termed this enzyme 3-phosphoinositide-dependent protein kinase-1 (PDK1). PIP3 binds to the pleckstrin homology (PH) domain of PKB altering its conformation so that Thr308 becomes accessible for phosphorylation. Since PIP3 is located at the plasma membrane of cells, the interaction of PKB with this molecule also recruits PKB to the plasma membrane, where it can be phosphorylated by PDK1, a significant proportion of which is present at this location. In addition, PIP3 binds to the PH domain of PDK1 which is critical for the activation of PKB in vitro and presumably also at the plasma membrane in vivo.

We and others have found that PDK1 phosphorylates and activates several other protein kinases in vitro which, like PKB, are members of the AGC subfamily of protein kinases. These include p70 S6 kinase, serum and glucocorticoid-induced protein kinase (SGK), MAP kinase-activated protein kinase-1 (MAPKAP-K1) also called p90rsk nearly all isoforms of protein kinase C (PKC) and cyclic AMP-dependent protein kinase (PKA).

In order to investigate whether PDK1 is really the enzyme that activates PKB in vivo and whether it is also required for the activation of other protein kinases in vivo, we produced embryonic stems (ES) cells in which both copies of the gene encoding PDK1 have been disrupted. These ES cells do not express PDK1, but are viable and proliferate at normal rates. The results of studies with the ES cells lacking PDK1 will be presented in the talk.

Regulation and role of the atypical PKCs in cell survival

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The two members of the atypical PKC subfamily of isozymes (aPKC), ζPKC and λ/ιPKC, have been implicated in the control of important cellular functions like cell proliferation and survival, most likely through the regulation of critical signaling pathways such as those that activate the AP-1 and NF-κB transcription factors. In this regard, the inhibition of the aPKCs severely impairs the activation of the MEK-ERK cascade by mitogens and the IKK system by TNFα. The understanding of the mechanisms whereby one given kinase can be involved in different cascades is not completely understood but could be related to the existence of specific anchors/scaffolds that confer selectivity to the kinase's effects. In this regard both aPKCs, but not the classical or the novel isoforms, bind selectively to p62 which is neither a regulator nor a substrate of these kinases but that has a number of motifs consistent with its role as an adapter linking the aPKCs to receptor signaling complexes such as the RIP/TRADD/TNFα receptor [Sanz, 1999 #470]. Both aPKCs also bind the protein Par-4 that has been implicated in the induction of apoptosis. Thus, several stress pathways including the withdrawal of neurotrophic factors promote an induction of Par-4 levels and its subsequent interaction with the aPKCs which serves to inhibit their enzymatic activity. This leads to the down-regulation of the IKK/NF-κB system which may explain why the overexpression of Par-4 sensitizes cells to TNFα-induced cell death. Interestingly, Par-4 levels are down-regulated in Ras transform cells. We demonstrate that the expression of oncogenic Ras reduces Par-4 protein and mRNA levels through a MEK-dependent pathway. In addition, the expression of permanently active mutants of MEK, Raf-1, or ζPKC but not of PI 3-kinase, is sufficient to decrease Par-4 levels. These effects are independent of p53, p16 and p19, and were detected not only in fibroblast primary cultures but also in NIH-3T3 and HeLa cells, indicating that they are not secondary to Ras actions on cell cycle regulation. Importantly, the restoration of Par-4 levels to normal values in Ras-transformants not only makes these cells sensitive to the pro-apoptotic actions of TNFα under conditions in which PI 3-kinase is inhibited but also severely impairs colony formation in soft agar and tumor development in nude mice, as well as the sensitivity of these tumors to chemotherapeutic agents. This indicates that the down-regulation of Par-4 by oncogenic Ras is a critical event in tumor progression. More recently the promoter of Par-4 has been cloned in our laboratory and the potential elements involved in its down-regulation are being characterized.

Structure, function, and regulation of MAP kinase pathways

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MAP kinase cascades are among the signal transduction pathways most commonly used to mediate intracellular responses to extracellular signals and environmental cues (English et al., 1999). Each MAP kinase cascade includes a minimum of three protein kinases activated in series. MAP kinases are activated by their specific upstream activating enzymes, MEKs, by phosphorylation on two closely spaced residues, a threonine and a tyrosine. The crystal structures of the MAP kinase ERK2 in the unphosphorylated and phosphorylated states explain how the two phosphorylation events increase the activity of the enzyme and reveal other interesting features of this enzyme family (Zhang et al., 1994; Canagarajah et al., 1997). We have used several strategies to understand the relationship of subcellular localization to function of ERK2 and the mechanism of its nuclear translocation (Khokhlatchev et al., 1998). We conclude that MAP kinases homodimerize following phosphorylation. Homodimerization contributes to the nuclear accumulation of active forms of these proteins. ERK2-MEK1 fusion proteins have elevated protein kinase activity, do not activate endogenous MAP kinases, and can be localized to individual subcellular compartments by the deletion or addition of targeting sequences. Their localization in the nucleus appears to be required to induce several transcriptional and phenotypic effects including cell proliferation (Robinson et al., 1998). The orphan MAP kinase ERK5, like ERK1 and ERK2, may also be important for cell proliferation. Dominant negative mutants of ERK5 and its upstream activator MEK5 block transformation induced by Raf-1, consistent with additional signaling pathways downstream of Raf (English et al., 1999).

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NIK recruitment to phosphotyrosine-containing proteins by Nck is an important event in the downstream cascade of EphB receptors that leads to JNK and integrin activation.

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The ste20 kinase NIK specifically activates the JNK module in mammals. NIK also binds the SH3 domains of the SH2/SH3 adaptor protein Nck. In search for upstream regulators of NIK, we found that NIK kinase activity is specifically increased in cells stimulated by EphB1 and EphB2. Eph receptors constitute the largest family of receptor tyrosine kinases and member of this family play important roles in patterning of the nervous and vascular systems.

Stimulation of EphB1 and EphB2 led predominantly to a complex between NIK and Nck, p62 dok, RasGAP and unidentified 145 kDa tyrosine phosphorylated protein. In searching for the identity of this protein, we found that the phosphatase SHIP2 is tyrosine phosphorylated in EphB stimulated cells. However, SHIP2 is not present in the complex with Nck and NIK.

Nik activation is critical for coupling EphB to biological responses as (I) it functions to increase EphB1 mediated attachment to fibrinogen, via integrin activation and (ii)it couples EphB to JNK activation.

Session 2: Mitogenic signalling (I)

Chair: Flora de Pablo

Mitogenic signalling and small GTPases

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Abnormal control of cell proliferation is a characteristic of tumours and so an understanding of the mechanisms controlling cell proliferation may provide new targets for cancer therapy. Small GTPases of the Ras and Rho families are involved in transmitting signals from growth factor receptors to intracellular signalling pathways controlling cell proliferation. Furthermore, mutated constitutively activated versions of these GTPases or their exchange factors can function as oncogenes. Several lines of evidence suggest that signals from Ras and Rho GTPases interact, but the molecular basis for this is not fully understood. One point of interaction is that some transmembrane receptors have to activate both Ras and Rho in order to get ERK MAP kinase activation. A second point of interaction is expression of the cyclin dependent kinase inhibitor $p21^{Waf1}$. When Rho signalling is blocked, activated Ras induces high levels of $p21^{Waf1}$ and fails to induce DNA synthesis. Consistent with these observations we have found that Swiss 3T3 cells transformed by oncogenic Ras have elevated levels of Rho GTP. When Rho is blocked in these cells $p21^{Waf1}$ is induced and the cells arrest. Thus Rho activity suppresses a signalling pathway of Ras that is required for transformation. While Rho GTP is elevated, these cells fail to respond to the Rho signal that leads to stress fibres, arguing that, in turn, Ras suppresses a signal from Rho.

While it is clear that activation of Ras is required for transmitting many mitogenic signals, it is not clear which Ras dependent signalling pathways are required. We have addressed this problem by using cells in which components of cell cycle control have been inactivated by homologous recombination. Using this approach we can show that loss of the tumour supressor pRb105 reduces the requirement for the ERK MAP kinase pathway for cell cycle re-entry. This suggests that the ERK MAP kinase pathway plays a major role in the activation of the CyclinD dependent kinases that phosphorylate and inactivate pRb105.

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Signaling by the insulin receptor

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Insulin is a key hormone implicated in a wide range of biological effects on metabolism, cellular growth, and differentiation. After insulin binds to the extracellular α-subunit of its receptor (IR), the tyrosine kinase activity of the β-subunit becomes active, leading to IR autophosphorylation and subsequently tyrosine phosphorylation of substrates. These tyrosine-phosphorylated molecules then act as docking proteins, recruiting SH2 domain-containing effectors such as phosphatidylinositol 3-kinase (PI 3-kinase), the adaptor growth factor receptor-bound protein-2 (Grb-2), and the phosphotyrosine phosphatase Shp-2.

In addition to IRS-1, the first identified bona fide substrate, several other direct substrates of the insulin receptor have been isolated including the different Shc isoforms, IRS-2,3,4 and Gab-1. To the growing list of insulin receptor substrates we have added recently using yeast two-hybrid and mammalian transfection studies Stat 5B, which belong to the family of SH2-domain containing transcription factors. We showed that Stat 5B interacts directly with its SH2-domain to the juxtamembrane tyrosine 960 of the insulin receptor, which has to be phosphorylated to allow this interaction to occur. Importantly, we found that the insulin receptor phosphorylates Stat 5B on tyrosine 699, which allows Stat 5 dimerization and hence its activation. More recently, we have approached the biological relevance of the interaction between the insulin receptor and the transcription factor Stat 5B. Firstly, we show that both insulin and IGF-I lead to tyrosine phosphorylation of Stat 5B and this promotes binding of the transcription factor to the β-casein promoter containing a Stat 5 binding site. Further, we demonstrate that insulin stimulates the transcriptional activity of Stat 5B. Activation of Stat 5B by insulin appears to be Jak2-independent, whereas Jak2 is required for growth hormone-induced Stat 5B activation. Hence the pathway by which Stat 5B is activated by insulin is different from that used by growth hormone. In addition using Jak1- and Tyk2-deficient cells we exclude the involvement of both Jak1 and Tyk2 in Stat 5B activation by insulin. Taken together, our results strengthen the notion that insulin receptor can directly activate Stat 5B. More importantly, we have identified a Stat 5 binding site in the human hepatic glucokinase promoter and we show that insulin leads to a Stat 5B-dependent increase in transcription of a reporter gene carrying this promoter. These observations favor the idea that Stat 5B plays a role in mediating the expression of the glucokinase gene induced by insulin. As a whole these results provide evidence for the occurrence of a newly identified circuit in insulin signaling in which the cell surface receptor is directly linked to nuclear events through a transcription factor. Further, we have revealed an insulin target gene whose expression is, at least in part, dependent on Stat 5B activation and/or binding.

The cytokine Stat signaling pathway is negatively modulated by Socs proteins (Socs = suppressors of cytokine signaling). The best described of these proteins are Cis and SOC-1 (also named SSI-1 and JAB-1) to SOCS-7. They share an homologous structure, and N-terminal region of variable length, a central SH2 domain and a C-terminal SOCS box. The SOCS are rapidly induced by various cytokines, in a tissue-specific manner. Once expressed they participate in a negative feedback loop by inhibiting cytokine mediated Jak-Stat activation by various mechanisms.

Based on our demonstration that insulin leads to Stat5-mediated gene expression, we investigated whether the Socs pathway might be involved in controling insulin signaling. We found that in 3T3-L1 adipocytes, insulin induces SOCS-3 expression but not expression of Cis or SOCS-2. Using transfection of Cos-7 cells, we show that insulin-induction of SOCS-3 is dependent upon Stat5B expression. Moreover, Stat5B from insulin-stimulated cells binds directly to a Stat element present in the SOCS-3 promoter. Once induced, SOCS-3 blunts insulin activation of Stat5B, without modifying the insulin receptor tyrosine kinase activity. This negative regulation likely results from competition between SOCS-3 and Stat5B binding to the same insulin receptor motif. Indeed, using a yeast two hybrid system we show hat SOCS-3 binds to the insulin receptor at phosphotyrosine 960, which is precisely where Stat5B binds. This association was confirmed in intact cells using confocal microscopy. Indeed, in unstimulated cells SOCS-3 is found throughout the cytoplasm. Importantly, insulin induces colocalization of insulin receptor and SOCS-3 at the cell membrane. This translocation requires tyrosine 960 of the insulin receptor since with cells expressing receptors mutated on Y960 (Y960F) no insulin-induced SOCS-3 translocation is seen.

To conclude, we have thus revealed a second insulin-target gene of which the expression is dependent upon Stat5B activation. Importantly, SOCS-3 for by inhibiting insulin-stimulated Stat5B, SOCS-3 appears to function as a negative regulator of insulin signaling.

Osmo-stress signal transduction in *S. cerevisiae*

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The adaptive response to high osmolarity is crucial for yeast cells as their natural habitats pose the potential for extreme osmotic conditions. When yeast cells detect changes in extracellular osmolarity they respond to it through activation of a MAP kinase pathway. Of several osmo-adaptive mechanisms, accumulation of compatible osmolytes such as glycerol appears most important [1], that is why this pathway is frequently referred to as the HOG (high osmolarity glycerol response) pathway. Induction of stress adaptive genes such as GPD1 (glycerol-3-phosphate dehydrogenase) is regulated by the HOG pathway and occurs following the detection of hyperosmolarity by either of two independent osmo-sensors, Sln1 and Sho1.

The first osmosensor, Sln1, is similar to the prokaryotic two-component signal transducers [2]. The Sln1 osmosensor requires two other molecules, Ypd1 and Ssk1, for its function, and this Sln1-Ypd1-Ssk1 system constitutes a multi-step phospho-relay mechanism [3]. When unphosphorylated Ssk1 acts as an activator of a MAPK cascade. This kinase cascade is composed of a pair of redundant MAPKKKs (Ssk2 and Ssk22), MAPKK (Pbs2), and MAPK (Hog1). Binding of Ssk1 to the amino-terminal regulatory domain of Ssk2/Ssk22 induces activation of these kinases and this consequently results in activation of Pbs2 and Hog1 [4].

The second putative osmosensor Sho1 has four predicted transmembrane segments and a carboxyl-terminal cytoplasmic region containing an SH3 domain [5]. Sho1 interacts with, and activates, the Pbs2 MAPKK through Ste50 and the Ste11 MAPKKK [6]. Ste11 is a MAPKKK involved in other signaling pathways such as mating or invasive growth. However, these different pathways are normally well insulated from each other, probably due to the formation of multi-protein complexes. The two transmembrane osmosensors, Sln1 and Sho1, function independent of each other. Nonetheless, signals emanating from the two osmosensors converge at the Pbs2 MAPKK, which then phosphorylates the Hog1 MAPK. Phosphorylated Hog1 MAPK is translocated into the nucleus by a transport mechanism that requires the importin β homologs Nmd5 and Xpo1 [7]. Once inside the nucleus, Hog1 phosphorylates and activates transcription factors that induce glycerol synthesis and other adaptive responses.

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Pathways involved in the regulation of cell survival by oncogenes

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Detachment of untransformed adherent cells from extracellular matrix commonly triggers programmed cell death, a phenomenon termed anoikis. This is prevented by transformation of cells by a number of oncogenes, including Ras. In normal cells, matrix adhesion stimulates PI 3-kinase and hence the downstream kinase Akt/PKB to provide a suppression of death signal function. The protective effect of oncogenic Ras is transduced principally through its ability to activate PI 3-kinase and Akt.

The mechanism by which matrix adhesion activates PI 3-kinase activity has been explored by the use of fusion proteins of the pleckstrin homology domain of Akt with green fluorescent protein (GFP). GFP-PH Akt can be used to determine the sites of PI 3-kinase activation in the cell. On new adhesion of epithelial cells to matrix, PI 3-kinase activation occurs at actin and β-catenin rich structures known as podosomes. Focal contacts are not major sites of PI 3-kinase activation, suggesting that p125 FAK may not be involved in the activation of PI 3-kinase in this system. In addition, PI 3-kinase is strikingly activated at sites of cell-cell interaction, suggesting that these contacts also provide an anti-apoptotic signal.

Death is induced following detachment as a result of loss of survival signals to the cell. This allows a constitutive death inducing signal to predominate in the detached cells: this signal appears to emanate from death receptors such as Fas and DR4/5, and can be blocked by dominant negative FADD or over-expression of SODD, both inhibitors of death domain signalling. The detachment-induced death signal is manifested by rapid disturbance of pro-apoptotic Bcl-2 family protein function, followed by loss of cytochrome c from the mitochondria. Oncogene induced survival pathways impact at the earliest stage of this process.

There is a significant transcriptional component to the ability of Akt, and also other Ras effectors such as Raf, to influence cell death. In order to understand more about the mechanism whereby Akt and Raf can protect cells from apoptosis under different circumstances, we have used conditionally active forms of these kinases expressed in normal epithelial cells. Gene Chip technology has been used to study the ability of these kinases to regulate transcription of several thousand. This has revealed novel mechanisms for the regulation of apoptosis downstream of Ras. In particular, the ability of the Raf/MAP kinase pathway to induce autocrine expression of growth factors may account for the Akt-mediated protection of cells from apoptosis following strong activation of this pathway.

Phosphoinositide 3-kinase signaling

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Phosphoinositide 3-kinase (PI3K) is activated by a wide variety of growth factors, hormones, lymphokines and other cellular activators. It has been shown to contribute to cell survival, cell growth and cell migration.

A role for PI3K in human cancers has been inferred from the observation that the tumor suppresser gene, PTEN, is a phosphatase that dephosphorylates the 3 position of the inositol ring, thereby limiting the effects of PI3K in normal cells. The lipid products of PI3K can mediate recruitment of a variety of signaling proteins to the cell membrane and thereby initiate and coordinate complex cellular responses. Several downstream targets of PI3K have been identified, including two protein-Ser/Thr kinases, PDK1 and AKT (also called PKB) and protein-Tyr kinases of the Tec family (e.g. BTK). These proteins have pleckstrin homology domains that mediate binding to PtdIns-3,4-bisphosphate or PtdIns-3,4,5-trisphosphate in vitro. In order to understand the function of PI3K in vivo, we have deleted genes for subunits of PI3K in the mouse.

Deletion of all three isoforms of the p85 alpha subunit of PI3K results in mice that mostly die at birth, although a few p85 alpha -/- animals survive for several weeks and have been studied.

In contrast, mice deleted for p85 beta do not have significant defects in growth or development. The p85 alpha mice have a number of defects, including chylous ascites, abnormal glucose homeostasis and defects in B cell development and in B cell signaling.

The B cell defects are similar to those observed in mice with mutations in the BTK protein-Tyr kinase, consistent with BTK acting downstream of PI3K in mediating B cell activation.

This presentation will focus on regulation of phosphoinositide signaling and new information gained from disruption of lipid kinases in the mouse.

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Session 3: Mitogenic signalling (II)

Chair: George Thomas

Cellular signaling by tyrosine phosphorylation

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Tyrosine phosphorylation of cellular proteins plays an important role in the control of cell proliferation, differentiation, cell metabolism as well as other important cellular processes. All receptor protein tyrosine kinases (PTK) are composed of a large extracellular ligand binding domain, a single transmembrane domain and a cytoplasmic domain containing a conserved protein tyrosine kinase core flanked by regulatory sequences. Ligand binding to the extracellular ligand binding domain induces receptor dimerization. Dimerization of receptor tyrosine kinases is crucial for activation of the catalytic domain and for tyrosine autophosphorylation; both processes are mediated by an intermolecular process. Different ligands utilize different strategies for mediating receptor dimerization and activation. Growth hormone for example, binds simultaneously to two growth hormone receptors in a stoichiometry of 1:2. Namely, a single growth hormone molecule is able to bridge two growth hormone receptors. A large family of growth factors such as platelet derived growth factor (PDGF), stem cell factor (SCF), colony stimulating factor (CSF), and nerve growth factor (NGF) among many others are dimeric proteins. These growth factors induce receptor dimerization by virtue of their dimeric nature. Fibroblast growth factors on the other hand bind to their receptor monovalently, and when added alone are unable to induce dimerization and activation of FGF-receptors. It has been shown that FGF-induced dimerization of FGF-receptors is mediated by heparin sulfate proteoglycans. Biochemical, genetic and recent structural studies provide detailed pictures about the mechanism of action and activation of FGF-receptors in response to stimulation with FGF and heparin sulfate proteoglycans. In addition, recent kinetic and structural studies provided new insights as to how receptor dimerization activates the protein tyrosine kinase domain. For insulin or FGF-receptors it was shown that autophosphorylation of tyrosine residues in the activation loop releases an autoinhibition leading to stimulation of PTK activity. While key residues in the activation loop of insulin receptor interfere with ATP and substrate peptide binding, residues in the activation loop of FGF-receptor interfere with substrate, but not with ATP binding.

Modes of autoinhibition and activation in protein kinases

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The structures of autoinhibited forms of Pak1 and c-Src will be described and compared. In both cases, parts of the polypeptide chain outside of the kinase domain serve to induce or stabilize a conformational shift in the small lobe of the kinase, disrupting the catalytic sites in rather similar ways. The structures of the non-kinase segments are very different in the two cases, as are their likely interactions with activators -- Cdc42 or other molecules that bind with the so-called "CRIB domain" in the case of Pak1, SH3 or SH2 ligands in the case of c-Src. The conformation of the Cdc42-interacting part of Pak1 can be compared with that of the corresponding segment from Wiscott-Aldrich Syndrome protein (WASp), as seen in a published NMR structure of its complex with Cdc42 (Abdul-Manan et al, Nature 1999). We propose a network of regulatory interactions involving WASp (or N-WASp), Pak-like kinases, Cdc42 and (ultimately) the Arp2/3 complex.

Cloning of P220: A novel protein that interacts with Protein Kinase D/Protein Kinase C μ.

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The Protein kinase C (PKC) family of proteins plays a major role in signal transduction pathways and has been involved in diverse processes including growth, differentiation, changes in cell morphology, neural development, synaptic transmission, axonal regeneration, endocrine and exocrine secretion and tumour promotion. PKCs are activated by lipid second messengers, mainly diacylglicerol (DAG), in response to a variety of extracellular stimuli such as hormones, neurotransmitters, growth factors and cytokines. Protein Kinase D (PKD, also known as PKCμ) is a serine/threonine kinase distantly related to the PKC family, that contains a conserved DAG/phorbol ester-binding cysteine-rich domain but differs in enzymological and structural features from the PKC family members. PKD catalytic domain shares very little similarity to the highly conserved regions of the kinase subdomains of the other PKC members, suggesting that PKD is likely to have specific substrates and a different cellular function. In fact, we reported that PKD failed to phosphorylate several substrates which are actively phosphorylated by other members of the PKC family, showing an atypical substrate specificity in vitro. As yet, no substrate proteins of PKD have been found neither *in vivo* nor *in vitro*. In an effort to identify putative PKD/PKCμ specific substrates, we have used anti-PKD/PKCμ antibodies. We have immunoprecipitated, purified, sequenced and cloned a novel PKD interacting protein substrate, P220. We have generated the recombinant protein and specific antibodies against the C-terminal end of P220 that have enabled us to characterise the biochemical properties and localisation of this new PKD/PKCμ interacting protein. The further identification of P220 individual functions will enhance more our understanding of the sophisticated roles of PKD in cellular signaling.

Cytokine signaling; role of receptor associated proteins

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Hematopoiesis is regulated by cytokines such as erythropoietin (Epo) that interact with receptors of the cytokine receptor superfamily. These receptors associate with Jak kinases and mediate their activation. Once activated, Jaks phosphorylate the receptors as well as a variety of substrates including Stat factors. Epo receptor signaling induces the tyrosine phosphorylation and activation of the two highly related transcription factors Stat5a and b. However, mice containing a genetically alter, truncated receptor that no longer activates Stat5a/b have normal erythropoiesis. Moreover, detailed analysis of erythropoiesis in embryos and mice lacking Stat5a/b similarly fail to identify a critical role for Stat5a/b activation. The distal region of the Epo receptor also recruits PLC-(1 and PLC-(2 to the complex. Mice deficient in PLC-(2 show no defects in erythropoiesis but reveal an essential, non-redundant role for this enzyme in B cell receptor signaling downstream of Btk and Blnk as well as role in signaling through Fc receptor complexes including the collagen receptor on platelets. Many cytokines including Epo induce the expression of CIS/SOCS family members. Through SH2 domains CIS binds the Epo receptor while SOCS1 and SOCS3 bind Jak kinases through the tyrosine in the activation loop that is required for kinase activity. The significance of CIS induction is unclear since mice deficient in the gene have no phenotype. Conversely mice deficient in SOCS1 are born but die within 3-4 weeks. The perinatal lethality is eliminated by breeding the deficiency on the Rag2 deficient background demonstrating the essential role of T/B cell receptors. Studies support the concept that SOCS1 is critical in the regulation of the responses to several cytokines. Deficiency of SOCS3 is associated with embryonic lethality that is associated with an erythrocytosis and defects in erythroid lineage differentiation.

Receptor protein-tyrosine kinase and phosphatase signaling

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The stem cell factor (SCF) receptor protein-tyrosine kinase (PTK), c–Kit, is required for the proliferation, survival and differentiation of several types of stem cells, including hematopoietic stem cells, melanocyte stem cells, and germ cells. To investigate the function of individual tyrosine phosphorylation sites in the SCF receptor, we have used homologous recombination in ES cells to introduce mutations into individual phosphorylation sites in the c-Kit gene in the mouse germ line. We have generated mice carrying a Tyr719Phe mutation of Tyr719, which acts as a PI-3' kinase binding site. The mutation completely disrupts PI-3' kinase binding to the SCF receptor and reduces SCF-induced PI-3' kinase-dependent activation of Akt by >90%. These mice have apparently normal pigmentation and hematopoiesis, and females are fertile. However, F719/F719 males are sterile due to a defect in sperm development, which results from decreased spermatogonial stem cell proliferation and increased apoptosis (1). This indicates that SCF receptor signals through PI-3' kinase are critical for male germ cell proliferation and survival.

Receptor PTK signaling is transient, and a concerted series of processes turn off receptor signaling. One such process is the degradation of activated receptor PTKs. c-Cbl, an adaptor protein, which binds to a number of activated receptor PTKs, is a homologue of the C. elegans SLI-1 protein, which is implicated genetically in negative regulation of the LET-23 EGF receptor homologue in vulval development. c-Cbl has been shown to trigger polyubiquitination of activated receptor PTKs, a prerequisite for proteasome-mediated degradation. However, it was not known whether c-Cbl acted directly in the ubiquitination process, which involves a cascade of three enzymes, E1, E2 and E3. We have now shown that the RING finger domain of c-Cbl is able to activate the Ubc4 E2, and when linked to the c-Cbl "SH2" domain the RING domain stimulates ubiquitination of the activated PDGF receptor in the presence of E1 and the Ubc4 E2 (2). Thus, it appears that c-Cbl plays a direct role in mediating receptor PTK degradation by binding to the activated receptor via its "SH2" domain, and then through recruitment and activation of an E2 causing ubiquitination of the receptor. In this sense c-Cbl acts as an E3.

The activity of receptor-like protein-tyrosine phosphatases (PTPs) could in principle be regulated by ligands and play a role in transmembrane signaling. The membrane proximal catalytic domain of RPTPα can form a symmetrical dimer, in which a wedge from one monomer is inserted into the active site of the other monomer, suggesting that RPTPα activity be negatively regulated by dimerization. By enforcing dimerization through mutations that create disulfide bonded dimers (3), by crosslinking and FRET studies we have shown that RPTPα can dimerize constitutively in intact cells and that dimerization negatively regulates its catalytic activity in the cell.

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Session 4: Apoptosis and survival

Chair: Eric Wieschaus

PTEN: a tumor suppressor that functions as a phosphatidyl inositol phosphatase

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The tumor suppressor PTEN has been implicated in a large number of human tumors, and is conserved from humans to worms. Characterization of PTEN protein showed that it is a phosphatase that acts on 3-phosphorylated phosphoinositides, including PtdIns(3,4,5)P_3, and can therefore modulate signal transduction pathways that involve lipid second messengers. Recent results indicate that at least part of its role is to regulate the activity of the serine-threonine kinase AKT/PKB, and thus influence cell survival signaling.

Signal transduction mechanisms in the developing nervous system

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Extracellular stimuli elicit a variety of cellular responses that are critical for cell proliferation, differentiation, and survival during the development of the nervous system. The goal of our research is to identify the signal transduction pathways that mediate the responses of cells in the developing nervous system to neurotrophic factors and neurotransmitters. Recent studies that will be discussed have defined specific signaling pathways that promote neuronal survival and suppress apoptosis. We have characterized the mechanisms by which brain derived neurotrophic factor (BDNF) and its receptor tyrosine kinase TrkB promote neuronal survival. Activation of TrkB leads to the induction of an intricate signal transduction program. Of the many signaling pathways activated the phosphatidylisositol-3-kinase (PI3K)/Akt and the Ras/Erk signaling pathways play a critical role in promoting neuronal survival by both transcription independent and dependent mechanisms. Several targets of these signaling pathways have been identified that are critical regulators of cell survival and death including the Bcl2 family member BAD and the forkhead transcription factor FKHRL1. We will discuss our current findings regarding the mechanisms by which TrkB signaling leads to the inactivation of the pro-apoptotic functions of BAD and FKHRL1 and thereby promotes neuronal survival.

Identification of components of the cell death pathway

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Members of the tumor necrosis factor receptor (TNFR) family are related to each other in possessing extracellular cysteine-rich domains. A subset of these receptors share a cytoplasmic "death domain", a protein-protein interaction motif critical for engaging downstream components of the signal transduction machinery. The best studied death domain containing receptors are CD95 (Fas/Apo1) and TNFR1. Activation of either receptor by cognate ligand or agonist antibody can lead to cell death. New death domain containing family members include CAR1, a chicken cell surface receptor for cytopathic avian leukosis sarcoma viruses. The physiological ligand for CAR1 has yet to be identified. Another orphan receptor is mammalian death receptor 3 (DR3) also called WSL-1, Apo3, LARD and TRAMP. This receptor is highly expressed on thymocytes and lymphocytes and is likely to play a significant role in immune function. The other two death domain containing receptors DR4 and DR5 (Trick2/KILLER) are receptors for a novel member of the TNF-ligand family termed TRAIL or Apo2L. TRAIL is cytotoxic for a number of tumor cell lines and yet is relatively non-toxic for normal cell lines. Consistent with its non-toxicity, it is found to be constitutively expressed in many human tissues including spleen, thymus, prostate and lung.

Since DR4 and DR5 are expressed in both normal and transformed cells this raises the conundrum of why tumor cells are sensitive to TRAIL/Apo2L mediated apoptosis and normal tissues resistant? The answer lies in the existance of a third receptor (TRID/DcR1/LIT/TRAIL-R3) that functions as a non-signaling decoy receptor and is preferentially expressed in normal human tissues but not in most cancer cell lines.

The ramifications of these findings for cancer therapy are manifold and presently being explored.

From inflammation to skin biology: protein kinase cascades that control NF-κB activity

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Exposure to proinflammatory cytokines (TNF, IL-1), or byproducts of bacterial and viral infections, such as endotoxin or dsRNA results in activation of signaling cascades that eventually lead to stimulation of NF-κB activity and induction of NF-κB target genes. Such genes code for cytokines, chemokines, adhesion molecules and enzymes, such as $_{COX2}$, that produce secondary inflammatory mediators. Although chemically unrelated, the TNF and IL-1 receptors use members of the TRAF family (TRAF2 and TRAF6) as signal transducers that activate protein kinase cascades that regulate NF-κB activity by inducing the phosphorylation of IκBs is mediated by IKK, which is a complex protein kinase composed of two catalytic subunits (IKKα, IKKβ) and a regulatory subunit (IKKγ). IKKβ is the recipient of proinflammatory signals that are recruited to the complex via IKKγ and is directly responsible for IκB phosphorylation and NF-κB activation. By contrast, IKKα responds to developmental signals and it controls key steps in the differentiation of ectodermal derivatives, including the epidermis, cornea and conjunctiva. Although the physiological activators of IKK in response to different proinflammatory stimuli remain to be identified, not all of them have to function as IKK kinases. For instance, PKR which mediates the response to dsRNA activates IKK via protein-protein interaction rather than direct phosphorylation. In addition to positive control, IKK activity is subject to negative control. Some of the negative regulation of IKK activity is mediated via C-terminal autophosphorylation and some is due to a novel mechanism based on the synthesis of cyclopentenone prostaglandins (cyPGs). These compounds, whose synthesis is catalyzed by COX_2, react with a particular cysteine residue in the activation loop of the IKK catalytic subunits to form a Michael adduct. The same cysteine is also high reactive with thiol reagents and may form a unique site for control of IKK activity by changes in the intracellular levels of glutathione.

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Session 5: Signalling in cell differentiation and development

Chair: Joseph Schlessinger

Antiapoptotic signaling of the insulin/insulin receptor system in early neural development

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Programmed cell death is a well established developmental process in the nervous system. In contrast with the neuronal death induced by target-derived neurotrophins deprivation, the relevance of apoptosis in early neurogenesis and the identity of regulatory growth factors of local origin is poorly characterized. We have used the chick embryo during neurulation and the neuroretina during proliferative stages as models to study the relevance of early neural cell death and the *in vivo* survival role of (pro)insulin signaling.

We have shown that proinsulin mRNA is present, prior to the expression of IGF-I, in the neurulating embryo and the neuroretina, at stages when apoptosis is occurring with defined patterns. Insulin receptors are expressed in early neurogenesis, largely as part of insulin/IGF-I hybrid receptors with high affinity for insulin and proinsulin, as well as for IGF-I. *In vivo* treatment with antisense oligonucleotides or antibodies against proinsulin or the insulin receptor increases the frequency of dead cells in the locations where apoptosis preferentially occurs.

Treatment in culture with insulin also reduces the number of dead cells. In this system, insulin increases markedly the phosphorylation of Akt in both the neuroretina and the neurulating embryo, whereas ERK phosphorylation is only slightly affected by insulin in the neuroretina. In addition, c-Raf signaling is essential to maintain survival of young ganglion cells neuroblasts, as shown by *in vivo* infection with RCAS/ΔRaf, a c-Raf mutant form which blocks Ras-dependent Raf activation and induces an increase in apoptosis. Thus, embryonic (pro)insulin protects in vivo neuroepithelial cells and young neuroblasts from apoptosis in a process that involves most likely Akt. C-Raf appears implicated as well in the survival of young neuroblasts in neuroretina.

One unexpected molecule that may be also involved in the control of early neural survival is the chaperone Hsc-70, expressed in a developmentally regulated manner in the neurulating embryo and the neuroretina. The decrease in endogenous proinsulin induced by antisense oligonucleotides coincides with a decrease in Hsc 70 embryonic levels and with a parallel increase in apoptosis. Furthermore, treatment with antisense oligonucleotides to Hsc70 directly increases apoptosis. Hsc70 may be an endogenous stress-protective molecule during early developmental decisions that involve high risk of signal-dependent cell death.

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Genetic analysis of cell and body size in *Drosophila*

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The control of cell and organ size is critically important to the developing organism. The discovery of the Drosophila homolog of the vertebrate IRS1–4 (CHICO) has shown that manipulating the Drosophila insulin signaling pathway can lead to alterations in cell size, proliferation, and cellular metabolism. In vertebrates, the insulin signaling pathway activates two major branches: the Ras/MAPK pathway and the PI3K/PKB pathway. To determine the relative contribution of these two signaling pathways to the control cell size, mutations were created in a genomic rescue construct of *chico* in the PH and PTB domains as well as the Drk binding site and the two PI3K binding sites. Results obtained from this analysis and the characterization of mutations in genes coding for signaling components of the two pathways indicate that PI3K, PKB and PTEN play a critical role in the control of cell size and overall growth. To discover new genes involved in the control of cell size and growth, a screen for recessive cell size mutations in the eye was performed by making use of a tissue specific recombination system. If a *chico* mutant is made homozygous in the eye, then the entire head is small relative to the body. Thus this pinhead phenotype allows for the identification of genes involved in size control.

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PTEN$^{+/-}$ Mice: Molecular and Genetic Models of Carcinogenesis

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PTEN was identified as a tumor suppressor gene frequently deleted at chromosome 10q23 in a number of advanced tumors such as glioblastoma, prostate, breast, endometrial, kidney and thyroid carcinoma. Germline mutations of PTEN are believed to cause two related autosomal-dominant hamartoma syndromes: Cowden syndrome and Bannayan-Zonana syndrome. The affected patients share high susceptibility for development of benign hamartomas throughout the body in early life and predisposition to cancer. The human PTEN gene encodes a 403 amino acid polypeptide with a high degree of homology to protein phosphatases and tensin. Despite its homology to protein phosphatases, PTEN is able to dephosphorylate phosphatidylinositol (3,4,5) trisphosphate ($PI(3,4,5)P_3$), the primary product of phosphatidylinositol 3' kinase (PI3'K) activity and negatively regulate PI3'K-mediated cell survival.

Mice heterozygous for PTEN are highly susceptible to tumors. The predominant type of malignancies in PTEN$^{+/-}$ mice at a young age is of lymphoid origin. We present evidence that, past six months of age, PTEN heterozygous mice develop a range of tumors, some of which represent hallmark features of PTEN-associated hamartoma syndromes. The majority of PTEN$^{+/-}$ female mice develop breast and endometrial neoplasia, whereas males have increased incidence of neoplastic transformation of prostate epithelia. Significantly, the majority of tumors that develop in PTEN heterozygous mice are associated with LOH at the PTEN locus. Using a novel *in situ* approach we show that these tumors manifest hyperphosphorylation of PKB, providing a molecular mechanism for tumor formation in PTEN$^{+/-}$ mice. Thus, these mice represent a model system for the investigation of PTEN/PKB/Akt-related tumorigenesis in the laboratory. To investigate the potential interaction between PTEN and other tumor suppressor genes, PTEN$^{+/-}$ mice were bred into different genetic backgrounds and monitored for tumor formation. Mice heterozygous for both PTEN and p53, develop tumors at a significantly higher rate than the animals heterozygous for either of the genes, revealing a synergistic relationship between these two tumor suppressors in tumorigenesis. Preliminary evidence suggests a surprising functional relationship between PTEN and p53 and their respective roles in regulation of cell death and survival. A potential interaction between the p53 tumor suppressor gene and the PI3'K-dependent cellular survival signalling machinery is currently being investigated in hope of gaining mechanistic insight into the PTEN-p53 cooperation in tumor formation.

Role of cyclin D-dependent kinases *in vivo*

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Normal cell growth and differentiation requires precise control of the mechanisms that regulate the entry, progression and exit from the cell cycle. Entry of quiescent cells into the cell cycle is driven by the cyclin D-dependent kinases Cdk4 and Cdk6. Proper regulation of the activity of these kinases appears to be critical for normal cell proliferation since the majority of human tumors carry mutations that result in deregulation of these kinases.

The activity of Cdk4 and Cdk6 can be regulated at various levels:

(i) These kinases require a member of the cyclin family of proteins as a regulatory subunit. To date, only the three known D cyclins (D1, D2 and D3) are known to interact with and activate Cdk4 and Cdk6.
(ii) Cdk4 and Cdk6 are inhibited by the INK4 family of cell cycle regulators, P16INK4a, P15INK4b, P18INK4c and P19INK4d, which prevent cyclin D binding.
(iii) The catalytic activity of Cdk4/6-cyclin D complexes can also be inhibited by the Cip/Kip family of cell cycle inhibitors, P21Cip, P27Kip1 and P57Kip2. However, recent studies indicate that these inhibitors do not affect Cdk4/6 activity when bound at stochiometric ratios.
(iv) Cdk4 and Cdk6 are activated by phosphorylation in Thr161 by another cyclin-dependent kinase known as CAK (Cdk-activating kinase).
(v) On the other hand, Cdk4 and Cdk6 can be inhibited by phosphorylation by dual activity tyrosine kinases Wee1 and Myt1.
(vi) This phosphorylation process can be reversed by the Cdc25 family of tyrosine phosphatases.

In spite of this wealth of biochemical information, little is known regarding the role that these Cdks and their regulators play *in vivo*. During the last few years, various laboratories, including ours, have begun a systematic effort to generate mice carrying targeted mutations in these genes.

For instance, mice defective for cyclin D1 (ref. 1,2) are viable and fertile but small in size. These mice display severe retinopathy and their mammary epithelial cells fail to proliferate during pregnancy in spite of normal levels of ovarian hormones. In contrast, cyclin D2 is required for proliferation of ovarian granulosa cells in response to FSH whereas Cyclin D2 mutant male mice display hypoplastic testes (ref. 3). These studies illustrate the specific roles that these cell cycle regulatory molecules play *in vivo*. Further studies using double cyclin D1/D2 mutant mice will provide critical information regarding the role that these regulatory subunits play in those tissues in which they may have compensatory roles.

Genes encoding the four members of the INK4 family of cell cycle inhibitors have also been ablated in mice. INK4a mutant mice develop tumors with high frequency (ref. 4). However, the mutation present in these mice also eliminated expression of the tumor suppressor P19ARF. Thus, making it difficult to evaluate the contribution of P16INK4a to this phenotype. Mice lacking P18INK4c develop gigantism and widespread organomegaly. Their lymphocytes develop normally, but exhibit increased cellularity and a higher proliferative rate upon mitogenic stimulation (ref. 5). More importantly, loss of P18INK4c leads to the development of pituitary adenomas (ref. 5) as well as of other tumors (ref. 6, see below). More recently, mice lacking P19INK4d have also been described. These mice show limited testicular atrophy and do not have increased tumor development suggesting that P19INK4d in not a tumor suppressor (ref. 7).

In our laboratory, we have generated mice lacking either P15INK4b or P18INK4c proteins alone and in combination (ref. 6). Ablation of P15INK4b and P18INK4c results in similar

lymphoproliferative disorders and tumor formation, albeit P15INK4b mutant mice display a significantly lower incidence of neoplasias. In addition, mice lacking P18INK4c have deregulated epithelial cell growth that leads to the formation of cysts, mostly in the cortical region of the kidneys and the mammary epithelium. Concomitant loss of both P15INK4b and P18INKc does not result in significantly distinct phenotypic manifestations except for the appearance of cysts in additional tissues. These results indicate that P15INK4b and P18IKN4c are tumor suppressor proteins that act in different cellular lineages with limited compensatory roles (ref. 6).

We have also manipulated the Cdk4 locus to generate two additional strains of mice that either do not express Cdk4 [Cdk4 (neo/neo) mice] or express a mutant Cdk4 protein (Cdk4 R24C) that cannot bind the INK4 regulatory proteins (ref. 8). This mutation had been previously found to be associated with human hereditary melanoma (ref. 9). Mice devoid of Cdk4 expression are viable, but small in size and mostly infertile. The partial male sterility in cdk4 (neo/neo) mice is due to a block in late spermatogenesis. Female sterility is due to a defect in the formation of the corpus luteum. Cdk4 (neo/neo) mice also develop insulin-dependent diabetes due to a dramatic reduction in beta islet pancreatic cells. Mice expressing the mutant Cdk4 R24C protein are viable and fertile. These mice display hyperproliferative abnormalities, including the widespread formation of tumors. These results illustrate the distinct roles that cell cycle regulators play *in vivo* and how their mutation results in abnormal proliferation that leads, in most cases, to tumor development.

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The roles of Armadillo and APC proteins during *Drosophila* development

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Throughout *Drosophila* development, growth and patterning of epithelial cells depends on the stability and accumulation of the ß-catenin homologue, Armadillo. In the embryonic epidermis, ARM levels are controlled by Wnt signaling; a striped expression of wingless protein causes a striped accumulation of ARM. Uniform expression of mutant, stabilized forms of Arm cause embryonic phenotypes similar to those produced by ectopic Wnt. We show that some stabilized Arm mutant proteins produce their phenotype by driving endogenous wildtype Arm protein into the nucleus. This feature can be used to characterize the latent signaling capacity of mutant forms of Armadillo that are normally trapped in the cytoplasm.

In vertebrates ß-catenin level are also controlled by its association with the adenomatous polyposis coli protein (=APC). Mutations in the human APC tumor suppressor gene result in the development of colonic polyps and retinal lesions. There are two known APC homologues in flies. Mutations in one of these homologues (D-APC1) causes a loss of retinal photoreceptor neurons remarkably similar to the human retinal phenotype. This phenotype is thought to reflect a direct role for D-APC1 in regulating Arm activity. In cells, D-APC1 localizes to the minus end of microtubules, a intracellular localization thought to depend on a charged domain in the C-terminus of the protein. The human APC homologue associated with colon cancer has a similar domain in its C-terminus and shows a similar intracellular localization in tissue culture cells.

The second APC homologue in *Drosophila* (D-APC2) encodes a shorter protein that lacks the C-terminus. It does not localize to microtubules and shows a general cytoplasmic and cell surface localization in embryos. Based on their sequence, both D-APC1 and D-APC2 should still be capable of binding and downregulating Armadillo protein. We find that D-APC2 cannot substitute for APC-1 during retinal development. Instead, we show that deletions of D-APC2 modify the retinal phenotype of APC1. These experiments suggest that the two genes may have distinct roles in the regulation of Arm protein levels.

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POSTERS

TEMPORALLY REGULATED ASSEMBLY OF A DYNAMIC SIGNALING COMPLEX ASSOCIATED WITH THE ACTIVATED TCR

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T-cell antigen receptor (TCR) triggering promotes multiple tyrosine kinase-dependent interactions involving proteins with one or more protein binding modules. Reported interactions mostly exceed the binding potential of these proteins. A solution to this paradox is the temporally regulated recruitment of alternative ligands. We have tested this hypothesis by analyzing the dynamics of protein/protein interaction over a time course spanning the earliest events in the TCR signaling cascade. We show that a multimolecular, dynamic and short-lived complex is assembled on tyrosine phosphorylated CD3ζ following TCR engagement. The composition of this complex appears in a state of flux with regard to its individual components, which are recruited and shedded in a specific temporal sequence. We also show that protein/protein interactions within a subset of signaling proteins which play important roles in TCR signal transduction (ZAP-70, PLCγ, Vav. Cbl, Shc, SLP-76, LAT, Grb2), and are directly or indirectly recruited to activated TCR, are orchestrated in a temporal sequence specific and distinct for each of the proteins analyzed. The data provide a clue to the question of incompatibility of multiple interactions by placing these interactions in a temporal context. Furthermore, the formation of a complex higher order structure could explain how individual low specificity interactions can be coordinated into highly specific signaling pathways.

SELK-1, A TRUNCATED ISOFORM OF ELK-1 ORIGINATING FROM INTERNAL INITIATION, POTENTIATES NEURONAL DIFFERENTIATION.

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In cutured cells, multiple signals induce the MAP kinase ERK. The activated kinase translocates to the nucleus where it drives gene regulation through phosphorylation of the nuclear transcription factor Elk-1. Although Elk-1 is usually associated with cell proliferation and differentiation, this protein is also highly expressed in post-mitotic neuronal cells, where it is found in both nuclear and cytoplasmic compartments (Sgambato et al., 1998; **18**: 214-226). We now demonstrate the existence of a novel isoform of Elk-1 (45 kDa instead of 52 kDa), whose expression is restricted to neuronal tissues, including NGF-differentiated PC12 cells. This isoform, called sElk-1, arises from translational initiation at Met55, thus this protein lacks a portion of the amino-terminal, Ets binding domain. Despite this deletion, sElk-1 appears to bind weakly *in vitro* to the Serum Response Element (SRE) of the c-*fos* promoter together with SRF, but not with SRF deletion mutants that can form ternary complexes with wt Elk-1. Accordingly sElk-1 is a weak mediator of Ras-driven SRE activation in transient transfection assays. In PC12 cells overexpression of sElk-1 modifies the subcellular localization of Elk-1, causing it to relocalize predominantly in the cytoplasm instead of the nucleus. More importantly, sElk-1 overexpression significantly augments NGF-induced neuronal differentiation, an effect that is abolished by mutating the major MAPK phosphorylation sites at Ser383 and Ser389 in alanine. These data suggest that sElk-1 plays a crucial role in intracellular signaling leading to the regulation of genes implicated in neuronal differentiation.

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Insulin-like growth factor-II, phosphatidylinositol 3-kinase, nuclear factor-κB and inducible nitric oxide synthase define a common myogenic signaling pathway

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Insulin-like growth factors (IGFs) are potent inducers of skeletal muscle differentiation and phosphatidylinositol 3-kinase (PI 3-kinase) is an essential element for this process. However, the intracellular myogenic pathways activated by IGFs and PI 3-kinase remain to be defined. Here we show that IGF-II induces nuclear factor-kB (NF-kB) and nitric oxide synthase (NOS) activities downstream from PI 3-kinase and that these events are critical for myogenesis.

Differentiation of rat L6E9 myoblasts with IGF-II transiently induced both inducible nitric oxide synthase (iNOS) expression and NF-kB DNA-binding activity; these events correlated in time with the stimulation by IGF-II of nitric oxide (NO) production. Both IGF-II-induced iNOS expression and NO production were blocked by NF-kB inhibition. NF-kB or NOS inhibition totally abolished IGF-II ability to induce terminal differentiation in both rat and human myoblasts (myotube formation, expression of skeletal muscle proteins such as myosin heavy chain, GLUT4 and caveolin 3). Moreover, the NOS substrate L-Arg was able to mimic IGF-II-induced myogenesis and this effect was blocked by NOS inhibition.

Regarding the mechanisms involved in IGF-II activation of NF-kB, PI 3-kinase inhibition totally prevented NF-kB activation, iNOS expression and NO production. Moreover, IGF-II induced through a PI 3-kinase-dependent pathway, a decrease in IkB-a protein content that correlated with a decrease in the amount of IkB-a associated with p65 NF-kB.

In conclusion, we describe a myogenic signaling cascade initiated by IGF-II that leads to biochemical and morphological skeletal muscle cell differentiation and which involves (i) PI 3-kinase activation, (ii) IkB-a degradation and dissociation from p65 NF-kB, (iii) NF-kB activation and (iV) iNOS expression and activation. We also show the ability of the NOS substrate L-Arg to induce myogenesis in the absence of IGFs in both rat and human skeletal muscle cells which may be relevant in the treatment of myopathies.

Taking into account these data our next purpose will be focused on studying the role of the IKK (IkB kinase) complex in myogenesis.

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NGF activation of the extracellular-regulated kinase pathway is modulated by Ca^{2+}/calmodulin.

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Nerve growth factor is a member of the neurotrophin family of trophic factors that has been reported to be essential for the survival and development of sympathetic neurons and a subset of sensory neurons. Nerve growth factor exerts its effects mainly by the interaction with the specific receptor TrkA, which leads to the activation of several intracellular-signaling pathways. Once activated, TrkA also allows for a rapid and moderate increase of intracellular calcium, which would contribute to the effects triggered by nerve growth factor in neurons. In this report we wanted to analyze the relation of calcium and the activation of the Ras/extracellular-regulated kinase pathway in PC12 cells. We observed that calcium/calmodulin are both necessaries for the acute activation of extracellular-regulated kinases after TrkA stimulation. We analyzed the elements of the pathway that leads to this activation and we observed that calmodulin antagonists completely block the initial Raf-1 activation without affecting the function of upstream elements such as Ras, Grb2, Shc and Trk. We have broadened our study to other stimuli that activate extracellular-regulated kinases through tyrosine kinase receptors and we have observed that calmodulin also modulates the activation of this kind of kinases after epidermal growth factor receptor stimulation in PC12 cells and after TrkB stimulation in cultured chicken embryo motoneurons. Calmodulin seems to regulate the full activation of Raf-1 after Ras activation since a functional Ras is necessary for Raf-1 activation after NGF stimulation and CaM-Sepharose is able to precipitate Raf-1 in a calcium dependent manner.

PROTEIN KINASE SIGNALLING PATHWAYS INVOLVED IN THE SURVIVAL EFFECT OF EGF ON THE TGF-β-INDUCED APOPTOSIS IN FETAL HEPATOCYTES

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TGF-β-mediated apoptosis is one of the major death processes of hepatocytes. We have previously shown that TGF-β–induced apoptosis in fetal hepatocytes is preceded by the production of reactive oxygen species (ROS) (Sánchez et al, *J. Biol. Chem.* **271**, 7416-742, 1996). This process is dependent on protein synthesis (Sánchez et al, *Hepatology* **26**, 935-943, 1997). In this work, we have found that this factor disrupts the mitochondrial transmembrane potential ($\Delta\psi_m$) and activates the release of cytochrome c, with activation of caspase 3. We show here that TGF-β induces a decrease in the mRNA and protein levels of Bcl-x_L, an anti-apoptotic member of the Bcl-2 family capable of preventing cytochrome c release. The presence of radical scavengers block the decrease in Bcl-x_L levels, $\Delta\psi_m$ collapse and activation of caspase 3. Thus, the oxidative stress induced by TGF-β could mediate the decrease in Bcl-x_L levels and the induction of the mitochondrial permeability transition.

EGF, which is an important survival signal for TGF-β-induced apoptosis (Fabregat et al, *FEBS Lett.* **384**, 14-18, 1996), maintains Bcl-x_L levels, preventing the $\Delta\psi_m$ collapse, the release of cytochrome c and activation of caspase 3. The survival effect of EGF is not prevented by protein kinase C inhibitors. EGF activates p42- and p44- MAPKs, however, ERK inhibitors do not block the survival effect of EGF. Furthermore, phorbol esters, which activate both protein kinase C and MAPKs activities in fetal hepatocytes (Roncero et al, *Biochim. Biophys. Acta* **1012**, 320-324, 1998) have not survival effects on TGF-β-induced death. EGF also activates PI3K and PKB (AKT) in these cells. The presence of PI3K inhibitors block the protective effect of EGF on cell viability, caspase 3 activity, mitochondrial transmembrane potential disruption and Bcl-x_L levels. We conclude that PI3K mediates the survival effect of EGF on TGF-β-induced death by acting upstream the mitochondrial changes, i.e., preventing the transcriptional repression of bcl-x_L by TGF-β.

Protein kinase pathways involved in the survival of B-chronic lymphocytic leukemia cells.

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B-cell chronic lymphocytic leukemia (B-CLL) is characterized by the accumulation of long-lived $CD5^+$ B lymphocytes. Treatment with phorbol esters (TPA) inhibits spontaneous and chemotherapy-induced apoptosis in B-CLL cells. We have studied the signal transduction pathways involved in the inhibition of apoptosis by phorbol esters. The inhibition of apoptosis by TPA is mediated by the serine/threonine kinase PKC because two PKC selective inhibitors (GF109203X and Calphostin c) block this effect. Then, we used several selective inhibitors of different protein kinases in order to study the implication of each pathway in the survival effect induced by TPA. TPA induces ERK1/2 phosphorylation and PD98059, a selective inhibitor of MEK, blocks both ERK1/2 phosphorylation and the protective effect of TPA. LY294002, a selective inhibitor of PI3K, decreases the viability of B-CLL cells and partially blocks the effect of TPA. Furthermore, we have analyzed two PI3K-dependent pathways: TOR-p70S6K and Akt/PKB. Rapamycin, a selective inhibitor of TOR, has a slight effect on the viability of B-CLL cells. TPA induces PKC-dependent phosphorylation of Akt at serine 473 but not at threonine 308. Surprisingly, LY294002 does not affect TPA-induced phosphorylation of Akt. TPA also induces CREB phosphorylation and this phosphorylation is inhibited by GF109203X but is not affected by LY294002. Finally, TPA induces a decrease in the expression of IκB-α. This effect of TPA is not inhibited by LY294002 or PD98059, however is completely blocked by GF109203X.

PTP1B regulates insulin sensitivity and energy expenditure

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The cytoplasmic protein tyrosine phosphatase PTP1-B has been implicated in the negative regulation of insuli signaling. To explore the physiological role of PTP1-B, we targeted exon 1 by homologous recombination an generated PTP1-B null mice. PTP1-B -/- mice were born with the expected Mendelian ratio and were fertil When fed a regular chow diet, male, but not female PTP1-B knockout (KO) mice gained less weight then wil type (WT) littermates. When fed a high fat diet, both male and female KO mice gained less weight than W mice. The difference in body weight was due to decreased fat cell mass but not fat cell number in KO mic Food intake, on both diets, was not significantly different between WT and KO mice, nor did KO mice lose f through their GI tracts. Instead, KO mice exhibited significantly increased (~20%) energy expenditure and a increased respiratory quotient. Heterozygotes displayed an intermediate level of energy expenditure. Consister with these findings, leptin levels were low in KO mice, reflective of the decreased fat cell mass. As reporte previously for PTP1B KO mice deleted for exon 6 (Elchebly, M. et al), our KO mice also showed increase glucose tolerance, which was due to increased insulin sensitivity. Quantification by means of hyperinsulinemi euglycemic clamp showed that a two-fold higher glucose infusion rate was required in KO animals, with th bulk of this excess glucose being disposed of into muscle in KO mice. Interestingly, however, despite th decreased fat cell mass and enhanced insulin sensitivity, FFA levels were comparable in WT and KO mice. Ou results confirm that PTP1-B disruption confers insulin sensitivity and resistance to diet-induced obesity, an demosntrate for the first time that the latter is due to an increased in metabolic rate. Further work will b required to determine whether these effects represent actions of PTP-1B on a single or multiple phosphotyrosy proteins.

The activation loop of phosphatidylinositol phosphate kinases determines signaling specificity

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Phosphatidylinositol-4,5-bisphosphate plays a pivotal role in the regulation of cell proliferation and survival, cytoskeletal reorganization, and membrane trafficking. However, little is known about the temporal and spatial regulation of its synthesis. Higher eucaryotic cells have the potential to use two distinct pathways for the generation of $PI4,5P_2$. These pathways require two classes of PIP kinases, termed type I and type II. While related by sequence these kinases localize to different subcellular compartments, phosphorylate distinct substrates, and are functionally nonredundant. Here we show that a 20-25 amino acid loop spanning the catalytic site, termed the activation loop, determines both, enzymatic specificity and subcellular targeting of PIP kinases. Therefore, the activation loop controls signaling specificity and PIP kinase function and may be a target for signaling pathways that regulate $PI4,5P_2$ synthesis.

Role of the src-family of tyrosine kinases on Prolactin signal transduction

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Interaction of prolactin (PRL) with its receptor (PRLR) leads to activation of Jak and src family tyrosine kinases. The PRL/growth hormone (GH)/cytokine receptor family conserves a proline rich sequence in the cytoplasmic juxtamembrane region (Box 1) required for association and subsequent activation of Jaks. PRL addition to fibroblasts expressing the rat PRLR long form resulted in activation of c-Src and Jak2 and in tyrosine phosphorylation of the receptor. Receptor phosphorylation was due to associated Jak2, since in cells expressing either a Box 1 mutated PRLR (PRLR4P-A), unable to interact with Jak2, or a Jak2 with the kinase domain deleted (Jak2Δk), PRL did not stimulate receptor phosphorylation. Interestingly, addition of PRL to cells expressing PRLR4P-A resulted in an activation of c-src equivalent to that observed with the wild type receptor. These results demonstrate that PRL-mediated stimulation of c-src occurs independently of Jak2 activation and of receptor phosphorylation. In the human T47D breast cancer cells, PRL increases the phosphotyrosine content of FAK and paxillin. PRL stimulates c-src, fyn, FAK and JAK2 tyrosine kinase activities. The specific inhibitor of the src-family PP1, decreases both basal and PRL-induced FAK and paxillin tyrosine phosphorylation, but it does not inhibit JAK2 phosphorylation in response to PRL. In vitro, at the same concentrations, PP1 does not affect FAK or JAK2 kinase activities, indicating that the decrease of FAK tyrosine phosphorylation is secondary to a src-family inhibition, while the JAK2 kinase activity is independent of PP1 treatment. PRL also stimulates MAPK activity and the proliferation of the T47D cells. Both events are abolished by PP1 addition. These findings point to a central role for the src-family of tyrosine kinases in the proliferative and cell morphology changes induced by prolactin in T47D breast cancer cells.

THE TRKA IMMUNOGLUBULIN-LIKE LIGAND BINDING DOMAINS INHIBIT THE SPONTANEOUS ACTIVATION OF THE RECEPTOR

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Using domain deletion mutants of the TrkA extracellular domain, we have investigated the role of the two Ig-like domains in preventing receptor dimerisation and activation in the absence of ligand. We also generated chimeric receptors by replacing the first or the second Ig-like domains of TrkA with the third or fourth Ig-like domains of the c-Kit extracellular region, which are involved in ligand binding and dimerisation, respectively. Finally, amino acid substitutions were introduced in conserved residues within the two Ig-like domains of TrkA. These point mutations affected NGF binding in a way consisting with an important role for the the Ig-like structure in ligand binding. The biological activity of these mutant TrkA receptors was assayed by their ability to promote ligand-independent neurite outgrowth when transfected into PC12nnr5 cells, which lack endogenous TrkA. Rat-1 cells stably expressing these TrkA variants underwent malignant transformation as assessed by measuring proliferation in the absence of serum, anchorage-independent growth, and nude mice tumorigenesis. Biologically active mutant receptors exhibited spontaneous dimerization and constitutive tyrosine phosphorylation. These data indicate that the intact Ig-like domains inhibit TrkA activation in the absence of NGF.

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Interdependent promoter recruitment of a p38 like MAP kinase and its transcription factor targets in the context of stress induced genes in yeast

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In response to a hyperosmotic environment most cells activate a specific MAP kinase signal pathway. In S.cerevisiae this kinase, Hog1 induces a variety of genes related to osmo adaptation and general stress protection. It has been suggested that the kinase activates a variety of transcription factors by either controling their activation or DNA binding domain. By chromatin precipitation methods we show that the activated Hog1 kinase itself becomes intimately associated with the relevant promoter regions. Depending on the individual promoter MAP kinase activity can be necessary for the recruitement of both the target factor and the kinase. In other context even the catalytically inactive kinase is recruited to the promoter while remaining impotent for activation. In such a case however, the putative activator seems to be prebound at the promoter. Our findings highlight the possibility that a kinase can become an inherent part of the upstream activation factor perhaps not only targeting this activator but also general components of the transcripion initiation complex.

Protein phosphorylation in the modulation of G protein-coupled receptor signaling

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Activation of G protein-coupled receptors (GPCR) triggers the rapid phosphorylation of agonist-occupied receptors by a family of specific G protein-coupled receptor kinases (GRKs) and the subsequent binding to the phosphorylated receptors of regulatory proteins termed arrestins. This process results in GPCR uncoupling from G proteins (i.e., desensitization), and are also triggers receptor sequestration, dephosphorylation and recycling. Emerging evidence indicates that GRKs and arrestins are also directly involved in the pathway of activation of the MAPK cascade by GPCR.

Our laboratory is interested in investigating the mechanisms that regulate the localization, activity and cellular levels of GRKs and arrestins. In this context, we have recently identified new mechanisms that govern the activity and cellular expression levels of GRK2, an ubiquitous member of the GRK family, which phosphorylates a variety of GPCR. Our results indicate that the stability of GRK2 is tightly regulated through rapid degradation by the proteasome pathway. Activation of GPCR markedly increases GRK2 ubiquitination and degradation, suggesting that agonist-dependent modulation of GRK2 cellular complement is an important physiological step. In addition, activation of other signaling cascades can also induce rapid GRK2 degradation by additional proteolytic pathways.

On the other hand, recent evidence indicate that GPCR stimulation of the MAPK cascade involves recruitment and activation of cytosolic tyrosine kinases of the src family, probably using ß-arrestin as a src adapter protein. We have found that GRK2 can be modulated by src tyrosine kinase-mediated phosphorylation. GRK2 is a high affinity substrate for src "in vitro", and agonist stimulation of transfected $ß_2$-adrenergic receptors in Cos-7 cells leads to rapid "in situ" phosphorylation of GRK2 on tyrosine residues and results in enhanced GRK2 activity. Interestingly, activation of GPCR can also induce the rapid co-immunoprecipitation of MAPK and GRK2 and the negative modulation of GRK2 activity. These results indicate that GRK2 phosphorylation by c-src and MAPK is inherent to GPCR activation, and put forward new mechanisms for the feedback regulation of GPCR signaling to both G proteins and the MAPK cascade.

Heat Shock Protein 90 (Hsp90), a Scaffold for Creatine Kinase-B (CKB) in Thrombin Signaling?

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Protease Activated receptor-1 (PAR-1) is a seven-transmembrane G protein-coupled receptor that mediates cellular responses to thrombin during blood coagulation, cell proliferation, vascular injury changes, and tumor metastasis. Once activated by thrombin, PAR-1 induces rapid and dramatic changes in cell morphology. The signal is conveyed by a series of localized ATP-dependent reactions directed to the actin cytoskeleton. The mechanism and regulation of signaling events at PAR-1 however, is not clearly understood. Here we report that heat shock protein 90 (Hsp90), a molecular chaperone, and creatine kinase-B (CKB), a key ATP generating enzyme that regulates ATP within subcellular compartments, interact with the cytoplasmic-tail (C-tail) of PAR-1 both *in vitro* and *in vivo*. Disruption of the Hsp90/PAR-1 complex by the Hsp90-specific drug geldanamycin or reducing creatine kinase levels attenuated thrombin-mediated cell morphology/cytoskeletal changes. Geldanamycin and cyclocreatine (CKB inhibitor) also abrogated thrombin-induced RhoA activation without affecting calcium signaling. We also demonstrate that Hsp90 facilitates CKB binding to PAR-1 C-tail by interacting with the ATP generating enzyme. These results suggest that Hsp90 may function as a scaffold for CKB, which provides an ATP source for thrombin-mediated morphological changes. Present findings identify a novel mechanism for signal transduction in which Hsp90 may recruit molecule/s to the thrombin receptor complex.

Stimulation of nucleotide exchange by tyrosine kinases and inhibition of GAPs by PI3Ks cooperate to mediate Ras activation

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The intracellular guanosine triphosphate (GTP)-binding protein Ras has been established as a major regulator of cellular proliferation, differentiation and other cell functions. Ras activity is controlled by opposing effects of guanine nucleotide exchange factors (GEFs) and GTP-hydrolase (GTPase) activating proteins (GAPs). It is generally accepted that GEFs like Sos mediate agonist-dependent activation of Ras in a phosphotyrosine-dependent process. The activation of Ras by the epidermal growth factor (EGF) is probably the best understood system. Stimulation of the epidermal growth factor receptor promotes the recruitment of Sos together with adapter proteins to the activated receptor complex which facilitates Sos action on Ras. An EGF dependent increase in the guanine nucleotide exchange rate on Ras has been observed in permeabilized fibroblasts indicating that EGF does indeed activate Ras by stimulating GDP-for-GTP exchange on Ras through the membrane recruitment of Sos or a related GEF. Despite the existence of a large body of experimental data, however, the exact mechanism underlying Ras activation in most other systems remains obscure. We have investigated the role of tyrosine kinases in Ras activation in the myelomonocytic cell line U937. We found constitutive Ras activation in resting U937 cells which was sensitive to the tyrosine kinase inhibitors genistein and staurosporine. Detailed analysis revealed that both inhibitors abrogate Ras-GTP accumulation via inhibition of nucleotide exchange on Ras. Constitutive Ras-GTP formation in U937 cells was also sensitive to PI3K inhibitors. However, PI3K inhibitors did not alter nucleotide exchange on Ras but rather induced an increase in GAP action on Ras. Thus we find that both the activatory as well as the inactivatory branch of the Ras-GDP/GTP cycle are regulated in resting U937 cells: Tyrosine kinase(s) sustain nucleotide exchange and PI3K(s) allow Ras-GTP accumulation by inhibiting GAP protein(s).

We will also present initial data on the mechanism of agonist-induced Ras-activation in U937 cells and the involvement of protein kinases and PI3Ks in this process.

Regulation of the Peutz-Jeghers tumor suppressor kinase, LKB1, and identification of its potential substrates

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Peutz-Jeghers Syndrome (PJS) is an autosomal dominantly inherited cancer syndrome that has been recently shown to be due to inactivating mutations in a novel serine/ threonine kinase, LKB1. Inactivation of LKB1 represents the first example of a kinase acting as a classical tumor suppressor. Peutz-Jeghers patients are predisposed to intestinal hamartomatous polyps as well as a wide range of benign and malignant neoplasias. Interestingly, these and other clinical symptoms of PJS are highly overlapping with those of Cowden's Syndrome, which is due to inactivating mutations in the PTEN tumor suppressor. We have characterized a variety of upstream signals that regulate this novel kinase, focusing on a number of growth regulatory kinases. We have generated a series of mutants of LKB1 and analyzed their autophosphorylation, trans-phosphorylation, and growth suppressive functions. We have also initiated a number of approaches for identifying LKB1 substrates.

DIFFERENTIAL REGULATION OF SIGNALING CASCADES IN RESPONSE TO *HELICOBACTER PYLORI* INFECTION

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Infection with *Helicobacter pylori* (*Hp*) induces chronic gastritis, peptic ulcer disease, and even gastric carcinoma and lymphoma. Certain target genes activated by *Hp* are represented by histidine decarboxylase (HDC), COX-2 and proinflammatory cytokine promoters. The transcription factors NF-κB, AP-1 and GAS-RE-BP1/2 sign responsible for the activation of these genes. We have focused our attention on the differential activation of the MAPK signaling pathways induced by *Hp* in gastric epithelial cells leading to physiological disorders. AGS cells were infected with *Hp* and analyzed for activation of protein kinases belonging to the MAPK (ERK1/2) and SAPK (JNK) families. Transactivation studies, EMSA, in vitro phosphorylation assays combined with Western blot analysis using phosphospecific antibodies revealed that *Hp* mediates activation of the Raf-1→MEK1/2→ERK1/2 independent of H-Ras and MEKK1. Activation of the MKK4→JNK→AP-1 pathway by *Hp* infection was directed by PAK1 and the Rho-GTPases Cdc42 and Rac1. In contrast to the signaling pathway leading to activation of ERK1/2, the activation of the SAPKs was strongly dependent on adherence of the bacteria to the host cell and on the expression of virulence factors encoded by pathogenicity island (PAI) of *Hp*. Our data suggest that infection of host cells with *Hp* could induce activation of certain transcription factors by the induction of selected upstream signaling pathways. The identification of *Hp*-specific signaling pathways to inflammatory cytokine production or acid secretion casts a light on ways for drug intervention.

LIST OF INVITED SPEAKERS

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64 **Workshop on Novel Biocatalysts.**
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75 **1997 Annual Report.**

76 **Workshop on Initiation of Replication in Prokaryotic Extrachromosomal Elements.**
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103 **Workshop on the Neural Mechanisms of Addiction.**
Organizers: R. C. Malenka, E. J. Nestler and F. Rodríguez de Fonseca.

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105 **Workshop on the Molecules of Pain: Molecular Approaches to Pain Research.**
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