Instituto Juan March
de Estudios e Investigaciones

99 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Specificity in Ras and Rho-Mediated Signalling Events

Organized by

J. L. Bos, J. C. Lacal and A. Hall

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L. C. Cantley
J. G. Collard
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Introduction

J. C. Lacal

Ras and Rho are the founder-members of two families of small GTPases, each with more than ten members, which function in signal transduction from plasma membrane bound-receptors to cellular responses. The original "dogma" was that Ras and the Ras-like members control signalling cascades involved in cell growth and cell differentiation, whereas Rho-like GTPases control signalling towards cell architecture and motility. A substantial amount of research has focussed on these lines for the last 15 years. However, recently we have learned that signalling in which these small GTPases are involved, is much more broad and complicated than originally anticipated. Thus, there is now a large body of evidence demonstrating that Ras-like and Rho family members play a role in the control of transcription and the regulation of the cell cycle. Indeed, Ras and Rho proteins may impinge in a variety of cell responses such as cell growth and differentiation, senescence, cell death, development, motility, invasion and many others. Frequently, Ras and Rho GTPases co-operate in these processes by a complex network of cross-talking pathways. However, we have learned that each GTPase have its own peculiarities, with respect to structure, biochemical properties, localisation, etc. For instance, several Ras-like proteins have virtually similar effector domains as Ras, but their function appears to be completely different. A similar situation can be found with the Rho-like GTPases, with a large number of partners/effectors identified thus far. This is even further complicated by the still-growing number of potential effectors for each member of the Ras and Rho branches. Thus, it seems that besides a high degree of cross-talking among members of these two families of GTPases, there are still differences in the final biological end-points for specific members of the two families. Most effects measured thus far have been generated using vastly overexpressed systems. This is a valid approach to get an idea who is talking to who, and the relative hierarchy for each process, but the next step has to be more precise. This meeting has been called in an attempt to foster our understanding of the specific involvement of each family in biological end points, gathering a group of people who can specifically address the above issues from different sides.

Juan Carlos Lacal

Session 1: Concepts and specificity in signalling of Ras-like GTPases

Chair: Chris J. Marshall

Dynamic properties of Ras-like proteins and their importance for biological function

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GTP-binding protein functions as molecular switches which cycle between the GTP-bound ON-state and the GDP-bound OFF-state. They become activated by the action of guanine nucleotide exchange factors, GEF's, which increase the dissociation rate of nucleotide bound to the protein. The inactivation is achieved as a result of the intrinsically GTPase which is very slow and is catalyzed by GTPase-Activating Proteins, GAPs. In the GTP-bound state they interact with effectors or downstream targets which are defined as proteins interacting only, or rather much more strongly, with the GTP-bound active state.

Ras is the founding father of the superfamily of Ras-related proteins which contains a number of subfamilies the most important of which are the Ras, Rab, Rho, Ran and Arf subfamilies. They are proteins with a set of conserved sequence elements and a similar three-dimensional structure called the G domain. Another common feature of these proteins is that the principal structural difference between the GDP-bound and GTP-bound state is confined to small localized areas called the switch I and switch II, whose structural change is triggered by the presence or absence of the γ-phosphate group. These regions are usually conformationally flexible. In addition these regions are involved in almost all the interactions of the GTP-binding protein with regulators and effectors.

Focussing on Ras, investigations on the flexibility of the switch regions and their interactions with other proteins will be presented. NMR and X-ray structural studies show that the switch regions can be found in different, usually fast exchanging conformations. In combination with biochemical and mechanistic studies on the interaction of such regions with GAP, GEF and effectors these studies show that the conformational equilibrium of Ras is a central important feature of the biological function of the protein. Comparison with the nuclear GTP-binding protein Ran indicates that this maybe a general feature of Ras-like proteins.

Interconnectivity between the small GTPases Ras, Ral and Rap1

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Ras is the paradigm of a family of 13 small GTPases that share similarities in the effector domain, suggesting a relationship in function. For Ras, this function has been studied extensively, revealing a role for Ras in many biological processes. Ral is another member of the Ras family, of which the function is still largely elusive. Recently, it was found that Ral-specific guanine nucleotide exchange factors (RalGEFs) are direct effectors of Ras. Indeed, Ral mediates signals from Ras towards the induction of gene expression and cell cycle progression. For full oncogenic transformation the RalGEF pathway co-operates with other Ras effector proteins, i.e. Raf1 and phosphatidylinositol-3 kinase (PI-3K) [1]. We recently identified an interesting connection between the Ral and the PI-3K pathway in the transcription factor AFX. This factor is directly phosphorylated by PKB, a downstream target of PI-3K and by a putative kinase induced by the Ras-RalGEF-Ral pathway [2].
Another member of the family, Rap1 (or K-rev1) was identified as a revertant of Ras-induced cell transformation. This resulted in the hypothesis that Rap1 may be an antagonist of Ras signalling, but more recent evidence indicates that Rap1 is involved in a pathway distinct from Ras. Rap1 is very rapidly activated by a large variety of growth factors, suggesting some common function in signal transduction. Several Rap1-specific GEFs have been identified, one of which, Epac, was found to be directly regulated by cAMP, providing a novel cAMP-target protein [3].

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Ras signaling and growth control

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Ras proteins are key intermediates in the signaling pathways initiated by receptor tyrosine kinases. The activation of Ras following growth factor stimulation relies on a highly conserved mechanism involving the ligand-dependent assembly of protein complexes containing the Ras exchanger Sos. Sos consists of several defined domains each mediating a distinct function. The catalytic domain of Sos, located at the center of the molecule, specifically mediates guanine nucleotide exchange on Ras. The crystal structure of the catalytic domain of human Sos in complex with H-Ras has been solved recently. We have explored the mechanism of Sos-catalyzed nucleotide exchange by site-directed mutagenesis. This approach has led to the identification of a hydrophobic pocket on Sos as a primary binding site for Ras. In addition, specific mutations within switch I region of Ras and interacting residues of Sos impair the exchange activity of Sos thus revealing some key mechanistic features of the exchange reaction.

Once activated, Ras proteins trigger the activation of diverse signaling cascade through interactions with multiple effector molecules. We have examined the relationship between the intensity of Ras signaling and the cellular response. Normal levels of Ras signaling produce a mitogenic response which depends on the synergistic contribution of the ERK MAP kinase cascade and the Rac cascade. In contrast, expression of increasing amounts of the oncogenic form of human HRas, HRasV12, results in a dose-dependent induction of apoptosis in both primary and immortalized cells. The induction of apoptosis by HRasV12 is blocked by activated Rac and potentiated by dominant interfering Rac. The ability of Rac to suppress Ras-induced apoptosis is dependent on effector pathway(s) controlled by the insert region and is linked to the activation of NFkB and Rho. The apoptotic effect of HRasV12 requires the activation of both the ERK and JNK MAP kinase cascade and is independent of p53. These results demonstrate a role for Rac in controlling signals that are necessary for cell survival, and suggest a mechanism by which Rac activity can confer growth advantage to cells transformed by the *ras* oncogene.

Understanding signaling from Ras and Rho proteins in proliferation and apoptosis: identification of novel targets for anticancer drug design

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Malignant cells are characterized by a number of alterations in signal transduction pathways controlling proliferation, differentiation and apoptosis. The identification of these aberrant processes may allow the development of chemotherapeutic interventions to restore them or selectively destroy the transformed cells. However, and besides the great progress made in our understanding of the molecular mechanisms involved in cell transformation, only a few of the molecules identified as critical events in the carcinogenic process can be used as novel targets for intelligent drug design. Such molecules are usually found as key elements in signal transduction pathways regulating cell growth and apoptosis. We have focussed our efforts in understanding the mechanisms underlying cell growth and apoptosis where members of the Ras superfamily of oncogenes are involved. This will allow the identification of novel potential targets for anticancer drug design.

Ras proteins regulate different parallel signaling pathways, including at least Raf/MEK/ERK, PI3K/PKB and Ral-GDS/Ral/PC-PLD pathways. We have investigated the relationship of Ras proteins with the PC-PLD pathway. Several molecules derived as a consequence of membrane phospholipid breakdown are well established as second messengers in mitogenic signal transduction. Among them, phosphatidylcholine (PC) hydrolysis has been suggested to play an important role in mitogenesis and metastasis. PC is the most abundant membrane phospholipid in eukaryotic cells. Therefore, it can sustain a prolonged liberation of second messengers, without drastic changes in membrane phospholipid content. These long-lasting signals may be important in the acquisition of the transformed phenotype. Upon mitogenic stimulation by growth factors or oncogenic transformation, phospholipase D (PLD)-driven PC hydrolysis is activated, and both phosphatidic acid (PA) and choline (Cho) are generated. PA can be further hydrolyzed or deacylated to form DAG or lysoPA (LPA) respectively, both of them having mitogenic activity. Cho is converted into *P*Cho by choline kinase (ChoK).

We have demonstrated that ChoK is an essential event for the mitogenicity of growth factors such as platelet-derived growth factor (PDGF) or fibroblast growth factor (FGF) since they are unable to stimulate DNA synthesis if ChoK is inhibited. This observation is supported by the fact that the exogenous addition of *P*Cho to mouse fibroblasts induces the G1/S transition. Furthermore, we have demonstrated that *ras* oncogenes constitutively activates PC-PLD and ChoK. As a consequence, the basal level of phosphorylcholine (*P*Cho) is constitutively increased in *ras*-transformed cells. All these results justified the consideration of ChoK as a novel target for the development of anticancer drugs.

Generation of highly specific ChoK inhibitors allowed us to establish a good correlation between improvement in the inhibitory activity against the enzyme and the acquisition of antimitogenic potency *in vitro*. These new compounds had antiproliferative properties in the low micromolar range against oncogene-transformed cell lines and tumor-derived cell lines of human origin. Furthermore, these molecules were tolerated in mice at doses that showed *in vivo* antitumor activity against human tumor xenografts derived from HT-29 and A431 cell lines implanted subcutaneously in nude mice. This first generation of inhibitors provide *in vivo* evidence that blockade of *P*Cho production by inhibiting ChoK is a valid strategy for the development of new anticancer agents, opening a new avenue for the development of antitumor drugs with a novel mechanism of action.

The family of Rho proteins are involved also in a complex network of intracellular signals. We have reported that the human genes *rho* A, *rho* C and *rac*1 are capable of inducing apoptosis in different cell systems like NIH 3T3 fibroblasts and the human erythroleukemia K562 cell line after serum deprivation. Moreover, *vav* and *ost*, two guanine exchange factors for Rho proteins with oncogenic properties, were also able to induce apoptosis under similar conditions. We have investigated further the mechanism involved in this process and have demostrated that apoptosis induced by Rho proteins is independent of p53 but sensitive to expression of Bcl 2 protein under *in vivo* and *in vitro* conditions. Overexpression of *rho* induces the activation of an endogenous sphingomyelinase since apoptosis by human Rho A and Rho C proteins correlated with a decrease in sphingomyeline and an increase in ceramide levels, a putative second messenger for apoptosis. Furthermore, it was then verified that Rho-induced apoptosis is indeed mediated by generation of ceramides. We have seen also that Rho proteins play an important role in the physiological regulation of the apoptotic response to stress-inducing agents since a dominant negative mutant of Rac 1 interferes with the induction of apoptosis by TNFα.

Numerous groups have reported that both the JNK and p38 pathways can be activated by Rac and Cdc42. We have also demonstrated that the human Rho A, Rac-1 and Cdc42 proteins efficiently induce the transcriptional activity of nuclear factor κB (NF-κB) and that activation of Serum Response Factor (SRF) by Rho A is mediated by NF-κB and c/EBPβ transcription factors. These results strongly implicate Rho proteins in the regulation of signaling pathways leading to the nucleus. Both JNK and NF-κB have been proposed as dual signaling pathways that can be involved in either survival/transforming or apoptotic events.

In an effort to elucidate the intracellular mechanism underlying the induction of apoptosis by Rho GTPases, we have investigated further the apoptotic process induced by serum deprivation in NIH 3T3 cells stably overexpressing an activated version of the Rac1 protein. A mechanism that involves ceramides production, protein synthesis, as well as caspase-3 but not caspase-1 activation, and independent of cytochrome C release has been found. Rac1-induced apoptosis was related to the simultaneous increase in ceramide production and the synthesis of Fas-L. Generation of FasL is mediated by transcriptional regulation involving both JNK as well as NF-κB-dependent signals. None of these signals, ceramides or FasL, was sufficient to induce apoptosis in the parental cell line, NIH 3T3 cells. However, any of them was sufficient to induce apotosis in the Rac1 expressing cells. Thus, we conclude that Rac1 induces apoptosis by a complex mechanism involving the generation of ceramides and the *di novo* synthesis of FasL. These results suggest that apoptosis mediated by Rho proteins results from converging complementary signals including promotion and progression signals.

Apoptosis, or programmed cell death, is a genetically controlled, tightly regulated process in development and morphogenesis. Unregulated excessive apoptosis may be the cause of various degenerative diseases like Alzheimer or Parkinson, whereas an inappropiately low rate of apoptosis may promote survival and accumulation of abnormal cells that can give rise to autoinmune diseases and cancer. The involvement of Rho proteins in the regulation of both proliferation and apoptosis and the identification of the molecules involved will provide new targets for anticancer drug discovery such as those described above for *ras*-dependent signaling.

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Session 2: Ras and Rho signalling. What are the lessons from lower eukaryotes?

Chair: Alan Hall

GTPase signalling pathways in Drosophila – Rap1 and NF1

I. K. Hariharan, H. Asha, N. D. de Ruiter, M.-G. Wang, G. Cowley, S. Brill, A. Tchoudakova, I. The, J. Gusella* and A. Bernards

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Rap1

The normal biological function of the Rap family of GTPases is poorly understood. To understand the function of *Rap1 in vivo*, we have studied the phenotype of loss-of-function mutations in *Rap1* in Drosophila.

Our experiments do not support a role for *Rap1* as an antagonist of *Ras1* in vivo. *Ras1*-mediated signaling pathways in Drosophila are not influenced by changing *Rap1* levels and a reduction in *Rap1* levels does not phenocopy an increase in *Ras1* activity. Thus that *Ras1* and *Rap1* appear to have distinct functions *in vivo*.

Rap1 function is required during embryogenesis, imaginal disc development and oogenesis but seems unnecessary or is minimally required in the mitotically quiescent cells of adult Drosophila. Cell proliferation and cell fate specification appear to occur relatively normally in the absence of *Rap1* function. However, morphological aspects of differentiation are seriously impaired in the absence of *Rap1*. During embryogenesis, closure of the ventral furrow and head involution fail to occur when *Rap1* function is removed. Cell morphology is grossly abnormal in gastrulating embryos with cells displaying aberrant shapes and nuclear positions. Cell migrations are also significantly perturbed. At later developmental stages, the developing photoreceptor cells of the eye and the follicle cells of the ovary degenerate at a time when they normally undergo significant morphological change. All these findings point towards a role for *Rap1* in either directing or facilitating morphological aspects of differentiation.

NF1 (neurofibromatosis type 1)

Heterozygous loss of function mutations in the human NF1 gene result in a wide variety of manifestations including a predisposition to benign and malignant tumours, pigmentation abnormalities, short stature and learning disabilities. The precise mechanism by which mutations in the NF1 gene cause these various abnormalities is not well understood. The only known property of the NF1 protein is that includes a centrally located GAP-related domain (GRD). It is generally believed that loss of NF1 function results in increased Ras activity, which in turn promotes tumourigenesis.

We have generated null mutations in Drosophila NF1. Surprisingly these mutant animals are viable and fertile and show no overt abnormalities in Ras-mediated signalling. Thus NF1's function as a rasGAP may be redundant in vivo. However, the mutants have a significant growth defect; the larvae are 20-30% smaller than wild-type. Our studies indicate that the regulation of growth in the epidermis and the imaginal discs by NF1 may

reflect a requirement of the NF1 gene in the nervous system. Thus NF1 may act via a neuroendocrine pathways to regulate growth *in vivo*.

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ENRIQUE MARTÍN-BLANCO

GENETIC ANALYSIS OF DORSAL CLOSURE IN DROSOPHILA

Morphogenesis depends on the coordination of different cellular activities during development; cell division, growth, cell migration, changes of cell shape and biochemical differentiation. One of the best-studied morphogenetic processes is *Drosophila* embryonic dorsal closure. During dorsal closure the contralateral epidermal sheets meet at the dorsal midline. This process is driven by cell shape changes in the absence of cell proliferation.

Embryonic dorsal closure is driven by the activity of the *Drosophila* Jun kinase signaling cascade. This pathway becomes implemented by the Drosophila homologues of Rac1, Rho1, JNKK, JNK and c-Jun. Mutants for members of this signaling pathway show a dorsal hole in their cuticle and a lack of elongation of lateral epidermal cells. As a consequence of JNK activity, the expression of dpp, the Drosophila homologue of TGFb, is maintained at the most-dorsal epidermal cells. Remarkably, mutants for dpp receptors also present a dorsal hole phenotype. JNK and dpp signaling appear to be involved in the control of cytoskeleton dynamics, as this phenotype is shared by mutants for some cytoskeletal components.

By time lapse confocal microscopy we have found striking differences between JNK and dpp loss of function. JNKK mutations do not result in a lack of elongation of epidermal cells but in their detachment from the adjacent amnioserosa promoting the apoptosis of amnioserosa cells. In contrast, we found that in dpp receptor mutants the epidermal leading edge collapses, freezing the extension of the epidermal sheet. Furthermore, by interfering with dpp signaling in the amnioserosa, we have found a role for dpp maintaining the flexibility of the amnioserosa cells, which appears to be essential for completion of germ band retraction and dorsal closure.

Late in the Drosophila life, during metamorphosis, adult structures fuse in a process that parallels embryonic dorsal closure. However, it presents some clear morphological differences. This pupal closure implements the contralateral encounter of imaginal cells. Preliminary data coming from the analysis of wing imaginal discs suggest that epidermal cells substitute (not exclude) the larval tissue crawling over its surface. An analysis of the role of JNK signaling in this process is underway.

Regulation of cell integrity via Rho1 signaling

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Small GTPases play a crucial role in different aspects of *Saccharomyces cerevisiae* biology. One of the mechanisms by which these proteins control specific biological processes in this budding yeast is through the regulation of signaling through MAP kinase cascades. For example, both the Ras homologue Ras2 and the Rho-like GTPase Cdc42 are known to mediate the activation of the MAP kinase pathway controlling the pseudohyphal-invasive growth undergone by yeast cells under specific nutritional conditions. Cdc42 is also involved in the pheromone response pathway responsible for the mating process. Another Rho-like protein, Rho1, is essential for the activation of Pkc1, which controls a MAP kinase module consisting of the MAP kinase Slt2, which is activated by the redundant MAPK kinases Mkk1 and Mkk2, that are in turn activated by the MAPKK kinase Bck1 (1). This Pkc1-mediated pathway is responsible for the maintenance of cell integrity through the regulation of cell wall architecture during periods of polarized growth and in response to different environmental signals that lead to cell wall or plasma membrane stress. Transcription of a number of genes involved in cell wall biosynthesis has been shown to be dependent on this pathway, Furthermore, the inactivation of this pathway leads to a osmoticaly remediable lysis defect, indicating the importance of a functional pathway to ensure cell wall integrity (2).

In an attemp to gain insight into the function of Rho1 in signaling to the cell integrity pathway, we have exploited an anti-phospho MAP kinase antibody that specifically detect the active form of the MAP kinase Slt2 to examine the role of some elements operating in the Rho1 switch on the activation of the pathway. Our studies revealed that not only heat shock but also calcofluor white, caffeine and vanadate-induced Slt2 phosphorylation was blocked in a *rho1-104* strain, indicating that Rho1 plays a key role in activation of the cell integrity pathway in response to a variety of stimuli.

Rho1 activity is known to be upregulated by the GDP/GTP exchange factors (GEFs) Rom1 and Rom2, and downregulated by the GTPase activating proteins (GAPs) Sac7 and Bem2 (3). In addition, Bag7 shows a high identity to Sac7 and also contains a Rho-GAP domain. Whereas the involvement of Rom2, a GEF for Rho1, in controlling Rho1 activity in response to cell wall alterations has been shown recently, very little is known about how negative regulators of this small GTPase modulate signaling to the Slt2 MAPK cascade. Therefore, we analyzed the level of Slt2 phosphorylation in mutants affected in these proteins. The lack of Sac7 led to a strong increase in the level of phosphorylated Slt2 when cells were grown at 24°C. Consistent with the model in which the osmotic stabilization of the medium prevents Slt2 phosphorylation by physical stabilization of the cell surface, the presence of 1M sorbitol in the medium did not reduce the level of Slt2 phosphorylation in *sac7* mutants, in which activation of the pathway is not triggered from the cell surface but as a consequence of the constitutive activation of Rho1. Overexpression of the dual specificity phosphatase *MSG5* in a *SAC7* deleted strain abolished the Slt2 activation and partially suppressed the cold sensitivity of this mutant strain, indicative of this phenotype resulting from a constitutive activation of the Slt2 pathway. Disruption of Bem2 also gave rise to an increase in Slt2 phosphorylation but, in this case, the difference between the wild type strain and the *bem2* mutant was only seen when cells were grown at 30°C but not at 24°C. In contrast, the absence of Bag7 did not increase the level of active Slt2

at any tested temperature. Therefore, both Sac7 and Bem2 downregulate signaling to Slt2, although Bem2 presumably does not play a relevant role at 24°C. Additionally, the connection of other GTPase switches with the cell integrity pathway will be discussed.

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Cell wall stress depolarizes cell growth via hyperactivation of RHO1

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Saccharomyces cerevisiae cells respond to cell wall stress by transiently depolarizing the actin cytoskeleton (1). We report that cell wall stress also induces a transient depolarized distribution of the cell wall biosynthetic enzyme glucan synthase FKS1 and its regulatory subunit RHO1 (2). This redistribution of FKS1 and RHO1 from the growth site (bud) to the overall cell periphery is possibly a mechanism to repair general cell wall damage. The de- and repolarization of FKS1 is dependent on the actin cytoskeleton. Depolarization of the actin cytoskeleton and FKS1 is mediated by the plasma membrane protein WSC1, the RHO1 GTPase switch, the RHO1-effector PKC1, and a yet-to-be defined PKC1-effector branch (2,3). The stress-induced activation of RHO1 is independent of the PI kinase-related kinase TOR2, a previously characterized activator of the RHO1 switch (4,5). WSC1 behaves like a signal transducer or a stress-specific actin landmark that both controls and responds to the actin cytoskeleton, similar to the bidirectional signaling between integrin receptors and the actin cytoskeleton in mammalian cells. The PKC1-activated MAP kinase cascade is not required for depolarization, but rather for repolarization of the actin cytoskeleton and FKS1. Thus, activated RHO1 can mediate both polarized and depolarized cell growth via the same effector, PKC1, suggesting that RHO1 may have more than one 'on' position and my therefore function as a rheostat rather than as a simple on-off switch (2).

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The role of Cdc42p in polarity establishment during bud emergence and mating in yeast.

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In yeast, Cdc42p is required to induce cellular polarization at bud emergence and in response to pheromones. We are interested in the spatial and temporal regulation of Cdc42p. Activation of Cdc42p requires the G1 cyclin-dependent kinase (CDK) or the mating pheromone MAP kinase pathway which are thought to regulate the exchange factor Cdc24p. Interestingly, Cdc24p was found in the nucleus and activation of Cdc28p-Clnp triggered its relocalisation to the presumptive bud site. Nuclear localisation of Cdc24p requires Far1p; shortly before bud emergence, ubiquitin-dependent degradation of Far1p initiated by Cdc28p-Cln kinase triggers nuclear export of Cdc24p and bud emergence. Cdc24p was phosphorylated shortly after bud emergence and phosphorylation required Cla4p complexed with Cdc42p-GTP and the adaptor Bem1p. Available results suggest that phosphorylation of Cdc24p by Cla4p antagonized its function *in vivo*, suggesting that Cla4p is part of a negative feedback loop which turns off Cdc24p once polarisation has occured. During mating, cells orient polarized growth towards the mating partner using a pheromone gradient by a mechanism which requires Far1p and Cdc24p. Far1p binds Gβγ and also interacts with the polarity establishment proteins Cdc24p, Bem1p and Cdc42p, which in turn organize the actin cytoskeleton. In response to pheromones, the exportin Msn5p relocalizes Far1p together with Cdc24p from the nucleus to the cytoplasm, where it will interact with activated Gβγ. Thus, Far1p functions as an adaptor which recruits the polarity establishment proteins to the site of the incoming pheromone signal marked by Gβγ thereby polarizing assembly of the cytoskeleton in a morphogenetic gradient.

Session 3: Concepts and specificity in signalling of Rho-like GTPases

Chair: Lewis C. Cantley

Ras and Rho family GTPases promote co-ordinated cellular responses

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Rho, Rac and Cdc42 regulate membrane receptor-linked signal transduction pathways to the organization of the actin cytoskeleton. Once activated, Rho promotes the formation of focal adhesions (integrin complexes) and the assembly of actin stress fibres, Rac promotes both actin polymerization and integrin complex assembly at the plasma membrane leading to the formation of lamellipodia and Cdc42 triggers the formation of actin-rich filopodial extensions. In addition, these three GTPases have been reported to activate other cellular pathways including the JNK/p38 MAP kinases, the transcription factors NFkB and SRF, and the NADPH oxidase enzyme complex found in phagcoytic cells. It appears that Rho GTPases have the capacity to co-ordinately regulate changes in the actin cytoskeleton with changes in gene transcription. Ras family GTPases have been shown to regulate the ERK MAP kinase pathway, PI 3kinase activation and the activity of integrins. We are exploring the contributions made by Rho, Rac and Cdc42 and by members of the Ras family to cell movement, using a fibroblast wound healing assay and to phagocytosis using complement and immunoglobulin mediated receptor uptake in macrophages.

PI 3-kinase, Akt/PKB and Ras signalling pathways involved in the control of cell proliferation and survival

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Ras proteins act through direct interaction with a number of target effector enzymes, including Raf serine/threonine kinases, the type I phosphoinositide 3-OH kinases and Ral-GDS exchange factors. Oncogenic Ras has the ability to protect some cells, particularly those of epithelial lineages, from apoptosis. We have concentrated on understanding the mechanism involved in the induction of apoptosis by detachment of cells from extracellular matrix, and how this is prevented by Ras transformation. In normal cells, matrix adhesion stimulates PI 3-kinase and hence Akt/PKB to provide a suppression of death signal function. The protective effect of activated Ras is transduced through PI 3-kinase and Akt/PKB, without a major involvement of NF-κB. A number of substrates have been proposed to explain the protective effect of Akt/PKB, including the pro-apoptotic Bcl-2 family protein, BAD, caspase-9 and forkhead transcription factors. Evidence will be presented that these cannot account for all the protective effects of Akt/PKB, so other substrates must exist: a systematic search for uncharacterised physiological targets for Akt/PKB is being undertaken. Detachment induced apoptosis, also known as anoikis, involves the autocrine activation of Fas or related death receptors leading to stimulation of caspase 8 via a mechanism that is subject to feedback amplification from the mitochondria. Akt/PKB has differing impact on death receptor induced apoptosis depending on whether or not mitochondrial dysfunction is involved (Type I or Type II pathways).

In order to understand the mechanism by which adhesion to extracellular matrix induces activation of PI 3-kinase and Akt, we have made green fluorescent protein fusions with Akt/PKB. In living NIH 3T3 cells these fusion proteins translocate to membrane ruffles in response to stimuli that activate PI 3-kinase: this is blocked by PI 3-kinase inhibitors and by point mutations in the pleckstrin homology domain. In confluent epithelial cells such as MDCK cells, a high level of constitutive localisation of the GFP fusion proteins at the plasma membrane is seen, suggesting that these cells have high basal levels of PI 3-kinase activity. The GFP-Akt PH domain is found at the basolateral, but not apical, membranes of the cells in polarised culture. On attachment of suspended cells to matrix coated dishes, GFP-Akt PH domain localises to discrete sites at the basal surface that are rich in β-catenin, are quite distinct from focal adhesions, and appear to represent podosomes. In addition, strong localisation to sites of cell-cell interaction is seen, whereas membranes where cells are not in contact with their neighbours show no GFP-Akt PH localisation. These results suggest that cell-cell interaction results in local activation of PI 3-kinase in epithelial cells, and that a major part of the survival signal provided by cell attachment to matrix may be derived from cell-cell interactions in addition to cell-matrix interactions. Investigation of the mechanisms involved in cell adhesion induction of PI 3-kinase activation will be discussed.

EPS8 and E3B1 TRANSDUCE SIGNALS FROM RAS TO RAC

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The small guanine nucleotide (GTP)-binding protein, Rac, regulates mitogen-induced cytoskeletal changes, c-jun aminoterminal kinase (JNK) and its activity is required for Ras-mediated cell transformation. Epistatic analysis, using actin reorganization as a readout, placed Rac as a key downstream target in Ras signaling. However the biochemical mechanism regulating the cross-talk among these small GTP-binding proteins remains one of the major outstanding issues in signal transduction. In this study, we report that Eps8, a tyrosine kinase substrate, and E3b1/Abi-1, a protein associated to the SH3 domain of Eps8, are indispensable for the transduction of signals from Ras to Rac We show that: i) genetically-engineered removal of *eps*8 from cells results in the impairment of signal transduction from Ras to Rac; ii) Eps8, E3b1 and Sos-1 form a tri-complex *in vivo* which is required for the regulation of Rac-specfic Gef activities; iii) interference with E3b1 results in a phenotype indistinguishable from that of Eps8-nul cells. We propose a model in which Eps8 mediates the transfer of signals between Ras and Rac, by forming a complex with E3b1 and Sos-1.

Downstream signaling from Ras through the Ral-GTPases

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Ral proteins represent a distinct family of GTPases that show close similarity to Ras. A family of Ral-specific exchange factors have been shown to bind to and be activated by Ras. In addition, Ral-GEF activation has been shown to complement Ras induced cell proliferation in fibroblasts. These and other data support the idea that Ral-GEF and then Ral activation initiates a distinct downstream signaling pathway from Ras.

We have been investigating the contribution of Ral activation to growth factor and Ras signaling in PC12 cells. We have found that unlike in fibroblasts, where Ral complements other Ras effectors such as Raf and PI3 kinase, in PC12 cells Ral-GEF mediated signaling opposes Raf and PI3 kinase signaling (1). In particular, in PC12 cells Raf and PI3 kinase promote cell cycle arrest and neurite outgrowth, while Ral-GRF signaling promotes cell proliferation. As such, it inhibits neurite outgrowth induced by NGF or by other Ras effectors. Thus, the ratio of Ras effector activation may determine whether some cells divide or differentiate.

In PC12 cells we found that NGF induced only transient activation of Ral whereas it produces more chronic activation of Ras and Raf. These findings show that the ratio of Ras effector activation can change over time upon cell stimulation, and that the role of Ral activation is likely to delay the onset of NGF induced neurite outgrowth.

I plan to discuss the results of recent experiments aimed at understanding why Ral displays this anti-differentiation activity in PC12 cells.

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Small GTPases and cell proliferation

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Small GTPases of the Ras and Rho families are involved in transmitting signals to intracellular signalling pathways Activation of Ras has been shown to be essential for normal growth factor signalling and in a sizable fraction of some human tumours, particularly colon cancer and pancreatic cancer, Ras proteins are mutated to constitutively active forms. In many tumour cell lines which lack mutated oncogenic Ras, activation of normal Ras is required for proliferation. It is now clear that active Ras is capable of activating multiple signalling pathways These pathways include the ERK MAPkinase cascade via Ras dependent activation of the Raf protein, P13 kinase and guanine nucleotide exchange factors for the Ral GTPases[1]. In some contexts we have also shown that Ras activation plays a key role in activation of the p38 and JNK SAPK pathways. Inhibition of each of these Ras dependent signalling pathways, either by chemical inhibitors or through expression of interfering mutants, suppresses cell proliferation.

Cell proliferation is controlled through the regulation of cyclin dependent kinase activity. The activity of these kinases is regulated via multiple routes which include the control of phosphorylation of the kinases and the expression levels of cyclins and cyclin dependent kinase inhibitors. Signal transduction events must interface with the cell cycle machinery but much still needs to be learnt of how this takes place. We know that Ras signalling is required for growth factors to stimulate cell cycle entry but we still need to learn which cell cycle control points are regulated by Ras dependent signals? In normal cells a key target of the G1 Cyclin-Cdks (Cyclin D-Cdk4/6, Cyclin E-Cdk2) is the phosphorylation of the pRb105 tumour suppressor protein. Phosphorylation of pRb regulates cell cycle entry and occurs following growth factor stimulation. Ras dependent signalling is required for G1 Cyclin-Cdk activity and pRb phosphorylation because in cells in which pRb is deleted have reduced requirement for Ras dependent signalling[2]. Using inducible activated versions of Mek-1 and inhibitors of Ras dependent signalling it can be shown that the ERK-MAP kinase pathway and the P1-3 kinase pathway are required for CyclinD/Cdk4 activity[3].

As well as Ras, activation of Rho family GTPases has been shown to be required for growth factor induced cell proliferation. A key issue is to discover how Ras dependent signalling pathways and Rho family GTPases interact to control proliferation. Some growth factors require both a Ras and a Rho signal to activate ERKs. Furthermore in the case of the Ras dependent ERK cascade it is now clear that activation of this signalling pathway can either promote cell proliferation or suppress it through induction of the cyclin dependent kinase inhibitor p21Waf-1. We have shown that when Rho signalling is blocked, micro-injection of oncogenic Ras protein induces p21Waf-1 and cells do not go into DNA synthesis[4]. Rho signalling is therefore required to permit Ras to induce DNA synthesis. For non-transformed cells growing in culture serum growth factors provide the signal to activate Rho, however Ras transformed cells will grow in serum free medium. Furthermore the proliferation of Ras transformed cells in serum free medium is blocked by inhibiting Rho and this leads to elevated levels of p21Waf1. This suggests that in Ras transformed cells Rho becomes activated.

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Session 4: Biological end points of Ras-like GTPases

Chair: Alfred Wittinghofer

RGR, AN ONCOGENE LINKING RHO, RAL AND RAS MEDIATED SIGNAL TRANSDUCTION PATHWAYS. I. Hernandez-Muñoz, M. Malumbres, R. Diaz, P. Leonardi and A. Pellicer. Dept. of Pathology and Kaplan Cancer Center, New York University School of Medicine, New York.

Rgr is an oncogene that has been recently isolated in our laboratory by its ability to produce tumors in nude mice(1). It belongs to the GEF family, also called GDP-dissociation stimulator (GDS) family, and it has significant homology to RalGDS (Ral Guanine Dissociation Stimulator), a molecule that has been shown to be an effector of Ras and that it may functionally link Ras with other Ras-related proteins (2). Ras actions are mediated through interactions with multiple effectors (3). Through a kinase cascade Ras eventually activates Erk that then translocates to the nucleus where it phosphorylates and activates several transcription factors, including Elk-1. Elk-1 binds, together with the serum response factor (SRF), to the serum response element (SRE) within immediate early gene promoters (4, 5). Another pathway possibly activated by Ras includes MEKK1, a serine/threonine kinase, which is an upstream activator of SEK, which in turn activates JNK/SAPK (6). An accepted Ras effector is RalGDS, which activates, at least Ral proteins, that may in turn regulate the activity of two signaling molecules. One is RalBP1, a GAP protein for Cdc42 and Rac1, that binds to Ral-GTP (7). Another route where Ral might involved is in the activation of phospholipase D (3). Previous results of our lab suggest a connection of Rgr to the Ral pathway (1). Our analysis of the Rgr oncogene has focused on the signal transduction pathways induced by Rgr including proliferation, cell transformation and gene expression to understand its role in physiology and tumorigenesis. This protein that was, at least in part, activated by an N-terminal truncation, lacks the C-terminal domain found in other members of the family where these proteins are known to interact with the Ras gene products. The signal transduction pathways activated by this oncogene involve the Raf-MEK-ERK branch as well as Ral and Rho mediated pathways. In addition to the need for these three pathways to be intact to be able to exert transcriptional activation, the rgr oncogene produces in the host cells a rearranging of the cytoskeleton reminiscent of that produced by Rho (8), with increase of the actin stress fibers, and unlike that produced by Ras, which results in a disorganized actin cytoskeleton.
Mutational analysis of the oncogene indicates that additional N-terminal truncations that do not reach the catalytic region are still transforming, while point mutations at several positions in the catalytic region are sufficient to abolish the transforming phenotype. The results obtained open the way to analyze the crucial interactions between the Rho, Ral and Ras signal transduction pathways in the physiological context, as well as in malignant transformation.

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PI 3-Kinase signaling

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Phosphoinositide 3-kinase (PI3K) plays a role in regulation of cell growth, cell survival and cell migration by producing a set of phosphoinositides that regulate various signaling pathways. Research over the past ten years indicates that PI3K can act both upstream and downstream of ras family and rho/rac family G proteins. 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. In order to better 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. Several downstream targets of PI3K have been identified including two protein-Ser/Thr kinases, PDK1 and AKT (also called PKB) and a protein-Tyr kinase, BTK. These proteins have pleckstrin homology domains that mediate binding to PtdIns-3,4-bisphosphate or PtdIns-3,4,5-trisphosphate in vitro. This presentation will focus on signaling proteins that are activated by lipid products of PI3K and the effect of knocking out genes for PI3K subunits on development and signaling in the mouse.

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Regulation of integrin activity by R-Ras

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The ability of integrins to mediate cell attachment to extracellular matrices and to blood proteins is regulated from inside the cell. The level of ligand-binding activity of integrins is of critical importance in cell motility, cancer cell invasion, platelet aggregation and leukocyte extravasation to inflamed tissues. Our recent studies have shown that R-ras, a small intracellular GTP-binding protein, regulates the binding of integrins to their ligands outside the cell (Zhang et al., Cell 85: 61-69,1996). Transfecting non-adherent mouse (32D.3) or human (U937) myeloid cells with activated R-Ras (R-Ras38V) converted them into highly adherent cells. Conversely, a dominant negative R-Ras(R-Ras43N) transfected into adherent cells caused detachment of the transfected cells from the substrate. We have now found that the cell attachment-supporting activity of R-Ras is controlled by an Eph receptor tyrosine kinase, EphB2. Cells in which EphB2 is activated become poorly adherent to substrates coated with integrin ligands, and R-Ras is phosphorylated on tyrosine (J. Zou, B. Wang, A.H. Zisch, E.B. Pasquale and E. Ruoslahti, submitted). Transfections with R-Ras variants in which the critical phosphorylation site is mutated show that the R-Ras phosphorylation and loss of cell adhesion are causally related. We have also identified a candidate adapter molecule to connect R-Ras to EphB2. This R-Ras pathway is a unique regulatory pathway among the small GTPases. It may explain some of the physiological effects of the Eph receptors; the repulsive effect of these receptors in axonal pathfinding, and perhaps in cell migrations, may be caused by R-Ras-mediated reduction of substrate adherence.

The small GTP-binding protein R-Ras can influence integrin 'activation' by antagonising a Ras/Raf-initiated integrin suppression pathway. T. Sethi*, M.H. Ginsberg†, J. Downward¶, and P. E. Hughes†. *Department of Respiratory Medicine, University of Edinburgh Medical School, Teviot Place, Edinburgh UK. ¶Signal Transduction Laboratory, Imperial Cancer Research Fund, P.O. Box 123, 44 Lincolns Inn Fields, London WC2A 3PX, U.K. †Department of Vascular Biology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, USA.

The rapid modulation of ligand binding affinity ('activation') is a central property of the integrin family of cell adhesion receptors. The small GTP-binding protein Ras and its downstream effector kinase Raf-1 suppress integrin activation in a MAP kinase dependent manner. In this study we explored the relationship between Ras and the closely related small GTP-binding protein R-Ras in modulating integrin affinity state. We found that R-Ras does not appear to be a direct 'activator' of integrins in CHO cells. However, we observed that GTP-bound R-Ras strongly antagonises the Ras/Raf initiated integrin suppression pathway in these cells. Furthermore, this reversal of the Ras/Raf suppressor pathway does not appear to be via a competition between Ras and R-Ras for common downstream effectors, or via an inhibition of Ras/Raf induced MAP kinase activation. Thus, R-Ras and Ras may act in concert to regulate integrin affinity via the activation of distinct downstream effectors.

Regulation of Raf-1 by the p21 activated protein kinases (Pak). Mark Marshall*†, Huaiyu Sun*, Alastair King*, Bruce Diaz*† and Darlene Barnard†. Indiana University School of Medicine*, Indianapolis, IN, USA 46202. Lilly Research Laboratories, Indianapolis, IN, USA 46285†. Ras-dependent activation of the Raf-1 serine/threonine kinase can occur in response to growth factors, phorbol esters and expression of oncogenes. We have recently shown that the phosphorylation of serine 338 in the catalytic domain of Raf-1 by the p21 activated protein kinases is an essential step in the mechanism of Raf activation. Our initial findings suggested that the Cdc42/Rac pathway might feedback into the Ras/Raf pathway through Pak, presenting the possibility that one Ras effector pathway (Raf) might be regulated by a second Ras effector pathway (PI3 kinase). To further explore this hypothesis, we have examined the ability of Cdc42, Rac1, RhoA, Ras and mPak3 to stimulate the catalytic activity of a variety of Raf-1 mutants in COS-7 cells. Expression of activated forms of Cdc42 and Rac1 but not RhoA increased the basal activity of Raf-1 four to five-fold. When coexpressed with mPak3, Cdc42[V12] and Rac1[V12] further stimulated the basal activity of Raf-1 approximately 20 and 7-fold respectively. Cdc42[V12] and mPak3 were also observed to be potent stimulators of Raf-1 mutants defective for Ras binding (Raf[A84-87] and Raf-CR3). Identical results were obtained with an assortment of Raf-CaaX mutants. The membrane associated Raf-CaaX protein has a high constitutive activity, which is dependent upon the integrity of the Ras binding domain. The basal activity of Raf-CX is further increased several fold by the co-expression of Cdc42[V12] and mPak3. Interestingly, the low basal activities of the Raf-CaaX[A84-87] and Raf-CaaX[A84-87, S165,168] mutant proteins which lack the Ras binding sites were also fully activated by the combination of Cdc42[V12] and mPak3. As with normal Raf-1, expression of Cdc42[V12] alone also significantly stimulated the catalytic activity of all these mutant Raf-CaaX proteins. We next desired to determine if Ras itself played a role in promoting the Pak/Raf interaction independent of the physical association between Ras and Raf-1. When coexpressed with the membrane associated Raf-CaaX protein, Ras[V12] stimulated the constitutive activity of Raf-CaaX two to four-fold. Similarly, Ras[V12] stimulated the catalytic activity of the Ras binding mutants of Raf-CaaX to levels comparable with wild type Raf-CaaX. In addition, expression of a dominant-negative allele of mPak3 suppressed the Ras-dependent stimulation of the Raf-CaaX proteins. These results confirmed our hypothesis that an important consequence of colocalizing Ras and Raf-1 at the plasma membrane is the phosphorylation of Raf-1 serine 338 by Pak. Furthermore, the observation that Ras can stimulate Raf-1 activity through mPak3 without a physical association between Ras and Raf, suggests that Pak is being activated by a Ras-dependent pathway distinct from Raf, possibly PI3 kinase. Raf kinase activation experiments using effector-specific Ras mutants, such as Ras[C40] and Ras[S35] support a role for the regulation of Raf-1 by a Ras/PI3K/Pak pathway.

Ras in the control of cell senescence

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Normal, healthy, cells possess mechanisms that protect them from becoming neoplasically transformed by the simple action of oncogenes. Also, normal cells are unable to grow indefinitely in culture and, upon accumulation of a certain number of doublings, enter into a permanent growth arrest known as senescence.

The *INK4a-ARF* locus encodes two tumor suppressors, $p16^{INK4a}$ and $p19^{ARF}$, each regulating a different tumor suppressor pathway. Namely, $p16^{INK4a}$ activates Rb by inhibiting the CDK4 and CDK6 kinases; and $p19^{ARF}$ activates p53 by inhibiting the destabilizing effects of MDM2.

Expression of oncogenic Ras in primary cells is initially mitogenic but, after a period of a few days, triggers an anti-proliferative response mediated by both $p16^{INK4a}$ and $p19^{ARF}$ [1,2]. Cells arrested by introduction of oncogenic Ras are vey similar to senescent cells and, consequently, this phenomenon has been designated "premature senescence" [1]. We regard this response as an anti-tumoral mechanism that prevents the propagation of oncogenically-driven cells. Importanly, rodent cells genetically deficient in either $p16^{INK4a}$, $p19^{ARF}$ or p53 do not enter "premature senescence" upon introduction of oncogenic Ras but, on the contrary, they are efficiently transformed. This permissivity to Ras transformation was not observed with cells deficient in the cell-cycle regulators p21 [3] or p27 (unpublished observations).

In collaboration with Scott Lowe (Cold Spring Harbor, USA), we have found that the integrity of the Raf/MEK pathway is essential for Ras to elicit "premature senescence" [4].

We have begun a large-scale screening aimed at identifying other tumor-suppressor pathways that could be activated in response to the aberrant activity of oncogenic Ras. For this, we have used filters of high-density cDNA arrays (containing 18.000 ESTs) and we have compared the expression profile of cells arrested by serum deprivation with the expression profile of cells arrested by Ras-induced premature senescence. We will present the preliminary results obtained in this screening.

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Session 5: Specificity and biological end point of Rho-like GTPases

Chair: Angel Pellicer

Role of Rho GTPases in cell transformation and invasion

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The Rho Family GTP-binding proteins Rac, Rho and Cdc42 have been shown to control distinct features of the actin cytoskeleton. Over the past several years, we and others have shown that each of these GTPases plays an essential role in malignant transformation of Rat1 fibroblasts by oncogenic Ras (1,2). Analysis of the growth properties of Rat1 fibroblasts that express constitutively active mutants of Rac, Rho and Cdc42 and that of Ras transformed Rat1 fibroblasts that express dominant negative mutants of these GTPases, has indicated that these GTPases deliver distinct contributions to the transformation process. Thus, Rac appears to be predominantly involved in serum-independent growth, and also contributes to cell survival in the absence of serum, whereas Cdc42 plays a key role in anchorage-independent growth and integrin signaling (2,3). In addition, the functions of the Rho GTPases in the control of cell proliferation are likely to be independent of the roles that they play in the organization of the actin cytoskeleton.

We have also investigated the role of Rac, Cdc42 and Rho in motile behavior, using Rat1 fibroblasts that express dominant negative mutants of these GTPases. Interestingly, Rat1 cells that express either of these mutants are strongly inhibited in growth factor stimulated invasion in three-dimensional collagen gels, whereas growth factor stimulated locomotion on collagen surfaces is only inhibited in dominant negative Rac-expressing fibroblasts (4).

We are currently studying the role of Rho GTPases in the regulation of cell proliferation in MDCK cells, a dog epithelial cell line and are focussing on the control of cell survival in conditions of anchorage deprivation. Our results show that expression of constitutively activated mutants of either Rac or Cdc42 can rescue from suspension-induced apoptosis (anoikis). Interestingly, in the case of Cdc42, we observe a bi-phasic dependence of cell survival on the expression levels of constitutively active Cdc42-V12. At expression levels that are about five-fold lower than that of endogenous Cdc42, Cdc42-V12 protects against anoikis, whereas at expression levels of Cdc42-V12 that are similar or higher than that of endogenous Cdc42, this survival effect is abolished. In contrast to the effects of Rac and Cdc42, expression of constitutively active Rho stimulates anoikis. It is likely that the survival functions of Rac and Cdc42 contribute to cell transformation.

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Ras- and Rho-signaling networks in cell-adhesion and cell migration

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Rho-like GTPases, including Cdc42, Rac and RhoA, have been implicated in the control of a wide range of biological processes (1). In ,particular, Rho-like proteins act as key control molecules in signaling pathways that determine the reorganization of the actin cytoskeleton in response to receptor stimulation (2). Similar to Ras proteins, Rho-like proteins cycle between the active GTP-bound state and the inactive GDP-bound state. The dynamic cytoskeleton changes regulated by Rho-like GTPases determine the morphology, adhesion and motility of cells, processes required for the metastatic spread of tumor cells.

We have identified the invasion-inducing Tiam1 gene which encodes an activator (GEF) of the Rho-like GTPase Rac (3,4). Similar to constitutively active V12Rac, Tiam1 induces an invasive phenotype in T-lymphoma cells. Also activated V12Cdc42 induces invasion of T-lymphoma cells which is not caused by Cdc42-mediated activation of Rac but presumably by the stimulation of common downstream pathways of Cdc42 and Rac. Activated V14RhoA potentiates invasion but fails by itself to mimic Rac and Cdc42. The Rho-like GTPases thus cooperate in the acquisition of an invasive phenotype of lymphoid tumor cells (5).

In epithelial carcinoma cells, invasion and metastasis is often associated with reduced E-cadherin-mediated cell-cell adhesion (6.7). Ectopic expression of Tiam1 in epithelial cells inhibits HGF-induced cell scattering and cell migration by increasing E-cadherin-mediated adhesion. In addition, Tiam1-Rac signaling inhibits invasion and migration of fibroblastoid Ras-transformed MDCK cells by restoring E-cadherin-mediated adhesions and the epithelial phenotype (8). Interestingly, Tiam1/Rac-induced cellular responses with respect to cell-cell adhesion and cell migration are dependent on integrin-mediated cell substrate interactions. On fibronectin and laminin1, Tiam1/Rac signaling inhibits migration of MDCKf3 cells by restoring E-cadherin-mediated cell-cell adhesion, whereas on collagen Rac activation promotes motile behavior (9). Invasion and migration of epithelial cells is thus determined by a balance between invasion-inhibitory cell-cell interactions and invasion-promoting cell-substrate interactions, both mediated by PI3-kinase-regulated Tiam1-Rac signaling.

Using novel biochemical assays to determine the actual activation state of Rho-like GTPases (9, 10), we found that Rac and Rho play antagonistic roles in the establishment of the cellular and migratory phenotype of cells. Activation of Rac promotes cell spreading and cell-cell adhesions whereas activation of Rho leads to cell contraction and decreased cell-substrate interactions. In neuronal cells, the antagonistic role of Rac and Rho on the dynamic changes of the actinomyosin cytoskeleton may be regulated by Rac-mediated phosphorylation of the myosin II heavy chain and Rho-mediated phosphorylation of the myosin II light chain in neuronal cells (11). We found that Rac is also able to downregulate Rho activity directly at the level of the GTPase in different cell types. Transient PDGF-induced as well as sustained Rac activation by Tiam1 or V12Rac downregulate Rho activity in fibroblasts and epithelial cells. Neither LPA-induced nor constitutively active V14Rho affects Rac

activity, suggesting unidirectional signaling from Rac towards Rho. We conclude that Rac signaling is able to antagonize Rho directly at the GTPase level. The reciprocal balance between Rac and Rho activity determines cellular morphology and migratory behavior in epithelial and fibroblast cells.

The proto-oncogene Ras is frequently mutated in epithelial tumors (12), resulting in uncontrolled growth and transition towards an invasive, mesenchymal phenotype (6,7). Employing the novel biochemical assays (9,10), we have analyzed the activation state of Rac and Rho in response to oncogenic Ras signaling. Sustained oncogenic Ras signaling in epithelial cells permanently downregulates Rac and upregulates Rho activity which is accompanied by epithelial-mesenchymal transition. Oncogenic Ras provokes changes in Rac and Rho activity through sustained activation of the Raf/MAP-kinase pathway, which causes transcriptional downregulation of Tiam1, an activator of Rac. Reconstitution of Rac activity by exogenous expression of Tiam1 decreases Rho activity and restores the epithelial phenotype in mesenchymal V12Ras or RafCAAX-transformed cells. Our findings identify the inactivation of Rac by transcriptional downregulation of an exchange factor as an important mechanism for Ras-induced transformation of epithelial cells.

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RHO-MEDIATED ACTIN REORGANIZATION VIA LOCAL PROTEIN RECRUITMENT

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Many cellular processes are dependent upon actin cytoskeleton reorganization in response to various extra cellular stimuli. Rho GTP-binding proteins play an important role in signaling pathways that controls actin cytoskeletal organization, helping to generate actin-based structures such as filopodia, lamellipodia, and stress fibres.
In order to obtain experimental conditions reproducing a physiological situation after receptor engagement, we have developed a system that allows for the controlled and local recruitment of activated Rho proteins at discrete sites underneath the plasma membrane in living cells. This method is based on the ability of rapamycin to induce specific protein heterodimerization between the FK506 Binding Protein12 (FKBP) and the FKBP12-Rapamycin Binding (FRB) domain of the FKBP12-Rapamycin Associated Protein (FRAP). We have stably co-expressed in RBL cells (1) a plasma membrane protein which presents two FKBP domains as a cytoplasmic region together with (2) an activated, nonprenylated hence physiologically inactive form of Cdc42 to the FRB domain. By adding rapamycin to the medium, and by clustering the receptor using a bead-based system that allows for aggregation of the complex, we obtained the specific recruitment and local concentration of activated Rho proteins at the plasma membrane, bypassing any additional signaling proteins.
Local recruitment of activated Cdc42 and of its downstream effector WASP with the rapamycin system induced the formation of actin based membrane protrusions 1 to several µm long. These structures contain the cytoskeletal proteins VASP along the entire length and zyxin at the bead/membrane interface. We propose a model in which Cdc42, by means of its downstream effector WASP, recruits at specific membrane sites of receptor activation, zyxin that in turns binds VASP. VASP then brings profilactin to the site to promote actin polymerization. A differential distribution of ezrin, an actin-membrane linker, in the protrusions induced by CDC42 or by its effector WASP, indicates that filopodium and protrusion formation are the results of a multi-branched network of interactions that intersect at the level of Cdc42.
Recruitment of activated Rac1 to the plasma membrane with the rapamycin system triggered the internalization of up to 40% of the receptor clustering beads over a period of 2 hours. The internalization process was inhibited by cytocalasin D indicating that actin polymerization was required for uptake. Only very localized and limited ruffles were observable under the beads. Once inside, the beads to proceed a short distance into the cytoplasm. Bead uptake was inhibited by mutation of Leu37 in the effector region of Rac1 that prevents interaction with the downstream target POR1. This is the first demonstration that Rac-1 recruitment to the receptor site is sufficient to allow particle internalization in a process that resembles phagocytosis.

Regulation of neuronal morphology by Rho-kinase and its substrates

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Molecules such as extracellular matrix components, growth factors, and bioactive lipids can exert dramatic effects on neural architectures, ranging from stimulation of neurite outgrowth to induction of growth cone collapse and neurite retraction. Several investigators have reported that Rho regulates neurite retraction in N1E-115 cells or PC12 cells. In these cells, treatment with lysophosphatidic acid (LPA) leads to neurite retraction and growth cone collapse which is mediated by Rho. We previously identified Rho-kinase/ROK/ROCK as a downstream effector of Rho, and found that Rho-kinase elevates the level of phosphorylation of myosin light chain (MLC) leading to myosin II activation, and the activated myosin II is involved in the LPA-induced neurite retraction in N1E-115 cells downstream of Rho and Rho-kinase. These observations prompted us to explore neuron-specific substrates of Rho-kinase other than MLC. Here, we identified a novel substrate of Rho-kinase, collapsin response mediator protein-2 (CRMP-2), which was identified as a protein required for collapsin-induced growth cone collapse. CRMP-2 is also homologous to a C. elegans neuronal protein UNC-33 that is required for appropriately directed axon guidance. We identified the major phosphorylation site of CRMP-2 by Rho-kinase as Thr-555, and found that Rho-kinase phosphorylated CRMP-2 at Thr-555 in dorsal root ganglion neurons during the action of LPA. These results suggest that CRMP-2 is involved in axon guidance and in the regulation of growth cone morphology downstream of Rho and Rho-kinase.

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Ras-GRF an exchange factor in the midst of Ras and cdc42 GTPases

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Small GTP-binding proteins function as key molecular switches in the signal transduction routes that convey stimuli received by cell surface receptors to the nucleus. Among these, the Ras family of small GTP-binding proteins plays an essential role in the regulation of cell growth and differentiation, distributing signals to different downstream effector pathways including those mediated by mitogen-activated protein kinase (MAPK), PI-3 kinase and Ral-GDS. Ras GTPases cycle between an inactive GDP-bound and an active GTP-bound state and it is now known that this mechanism is controlled by at least two classes of regulatory proteins directly acting on Ras: GAPs (GTPase Activating Proteins), which potentiate of the capacity of the G-proteins to hydrolyze GTP, and GEFs (Guanine nucleotide Exchange Factors), that catalyze the exchange of GDP for GTP bound to Ras proteins, thus promoting their activation. $cdc25^{mn}$ /Ras-GRF is a GEF specific for the Ras family of small GTP-binding proteins cloned by virtue of its homology with the *Saccharomyces cerevisiae* CDC25 gene product, that stimulates nucleotide exchange on *S. cerevisiae* RAS. In mammals, Ras-GRF is expressed at high levels in brain but it has also been found to be expressed in other tissues and cell lines .

Analysis of the primary structure of Ras-GRF reveals the presence of a number of defined functional motifs presumably involved in different signaling control mechanisms and protein-protein interactions. As such, the carboxyl-terminal domain has strong homology with *S. cerevisiae* CDC25, thus its name, and it has been shown to catalyze nucleotide exchange on Ras. This domain is also present in other GEFs for the Ras family; SOS1 and SOS2, C3G, and GRF2. Ras-GRF also contains a Dbl-homology (DH) domain that bears a strong resemblance to the product of the *dbl* oncogene, a GEF for the GTPase cdc42. DH domains are generally present in GEFs for the Rho family of small G-proteins and other proteins such as ect2 of yet unidentified function but also believed to be involved in the regulation of the Rho family GTPases. In the case of Ras-GRF, the DH domain is flanked by two Pleckstrin homology (PH) domains, also found in most Ras and Rho family GEFs and other signaling proteins. PH domains are believed to bind phospholipids thereby playing an active role in membrane targeting .

Whereas Ras-GRF, it has been shown to be capable of inducing cellular transformation in fibroblast. Recent reports also suggest that its normal function may be to signal from G protein-coupled receptors to Ras, and that calcium may be implicated in its regulation by a mechanism involving a calmodulin-binding motif (IQ domain) near its N-terminus. Likewise, it has also been demonstrated that calcium can enhance Ras-GRF-mediated activation of the MAPK pathway.

Since the structural domains that constitute Ras-GRF are likely to play a distinct yet to be identified regulatory roles, we have constructed a series of deletion mutants for the different motifs to study their effects on Ras nucleotide exchange, MAPK activation and transformation. Our

results indicate that the DH domain is an essential feature for Ras-GRF-induced GDP/GTP exchange on Ras, MAPK stimulation and cellular transformation. In accordance, we have found that an inhibitory mutant of cdc42 can block Ras-GRF-induced nucleotide exchange on Ras, activation of MAPK and cellular transformation. Our data also indicates that while Ras-GRF is partially located in the particulate fraction, the DH domain mutant and Ras-GRF in the presence of cdc42 N17, are completely absent from this fraction. Interestingly, targeting Ras-GRF to the membrane could overcome the blockade exerted by cdc42 N17. In the same fashion, Ras-GRF DH domain mutant ability to activate MAPK is restored when sent to the membrane by a myristoilation signal. Thus, our data strongly suggest that Ras-GRF DH domain is an essential feature for targeting this exchange factor to the membrane by a yet unknown mechanism in which cdc42 plays a central role.

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POSTERS

A NOVEL PROTEIN RECRUITMENT SYSTEM FOR THE ANALYSIS OF PROTEIN-PROTEIN INTERACTIONS

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The yeast two hybrid system represents one of the most efficient approaches currently available for identifying and characterizing protein-protein interaction. Although very powerful, this procedure exhibits several problems and inherent limitations. Recently, the SRS (Sos recruitment system) was described. The SRS system is based on a different readout compared to the two hybrid system and overcomes several of its limitations. Thus, the SRS system serves as an attractive alternative for studying protein-protein interactions between known and novel proteins. Nevertheless, using the SRS system, we encountered a number of problems, and therefore developed an improved protein recruitment system.

The novel system, designated RRS (Ras recruitment system), is based on the absolute requirement of Ras plasma membrane localization for its function. Ras membrane localization and activation can be achieved through interaction between two hybrid proteins. We demonstrate the effectiveness of the novel RRS system using five different known protein interactions and identification of two novel protein interactions through a library screening protocol. The first is between JDP2, a member of the basic leucine zipper family (bZIP), and C/EBPγ, a member of the C/EBP family. The second is between the serine threonine kinase, Pak65 and a Cdc42 Homologue Protein, designated: Chp.
Recently, we have extended the use of the RRS system to a mammalian host cells as well using Ras responsive reporter assays. This improvement allows the verification and quantification of protein-protein interaction originally identified in yeast to be tested directly in mammalian cells. In addition, has the potential to serve as an approach for drug screening discovery to inhibit specific protein-protein interaction.
The RRS system significantly extends the usefulness of the previously described SRS system and overcomes several of its limitations thus can be used for identification of protein-protein interaction between known proteins and screening for novel interactions as well.

The role of the p21 activated kinases (Paks) in the phosphorylation and activation of Raf-1

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The Raf-1 protein is a serine/threonine specific protein kinase which couples Ras activation to the activation of the Erk mitogen activated protein (MAP) kinases. Raf-1 is normally cytosolic and inactive. Activation occurs through a complex process which is initiated by the Ras-dependent translocation of Raf-1 to the plasma membrane. However, this interaction between Ras and Raf-1 is not sufficient for activation and additional events, including Raf-1 phosphorylation and association with 14-3-3 are required for full activation.

We have recently shown that phosphorylation of S338 and Y341 of Raf-1 is required for full activation under a number of conditions [Mason et al (1999) EMBO J; 18: 2137]. We have developed phospho-specific antibodies to these residues and have shown that these phosphorylation events occur at the plasma membrane. Pak 3 (p21 activated kinase) is a serine/threonine specific protein kinase which is activated by the small GTP binding proteins Cdc42 and Rac. It has been shown that Pak3 can phosphorylate Raf-1 on S338 *in vitro* and it has been suggested that Raf-1 is a Pak3 substrate *in vivo* [King et al (1998) Nature 396:6707]. We have therefore examined whether Pak3 phosphorylates Raf-1 under physiological conditions.

We find that dominant negative versions of Pak3, Rac and Cdc42 do not block activation of Raf-1 by oncogenic Ras or activated Src. We find that activated versions of Pak3, Cdc42 and Rac do not stimulate Raf-1 activity when expressed alone and neither do they significantly enhance the activity of Raf-1 when co-expressed with oncogenic Ras or activated Src. Co-expression of wild type Pak3 with V12Cdc42 or V12Rac also does not lead to Raf-1 activation. Finally, wt Pak3 together with V12Cdc42 (or V12Rac) only co-operate very weakly with oncogenic Ras to activate Raf-1 (less than 2 fold).

Using our phospho-specific antibody, we have also examined the effects of Pak3, Cdc42 and Rac on Raf-1 phosphorylation at S338. Dominant negative Cdc42, Rac and Pak3 do not prevent oncogenic Ras and activated Src mediated phosphorylation of S338. V12Cdc42 and V12Rac do not stimulate the phosphorylation of Raf-1 on S338 and neither do they co-operate with oncogenic Ras to give enhanced S338 phosphorylation. Although activated Pak3 or wt Pak3 when co-expressed with V12Cdc42 or V12Rac do give a slight (less than is seen with Ras alone) stimulation of S338 phosphorylation, there is no associated Raf-1 activation as described above. Furthermore, this phosphorylation does not require Raf-1 to be membrane associated, because mutant Raf-1 which cannot interact with Ras and so cannot localise to the plasma membrane is still phosphorylated on S338 under these conditions. Also, dominant negative Ras does not block the activated Pak3 mediated phosphorylation of S338.

Taken together, these data suggest that Raf-1 is not a physiological substrate of Pak3. The data show that under extreme conditions, Pak3 is able to weakly phosphorylate Raf-1, but this is not associated with any increase in Raf-1 activity, probably because it occurs in the cytosol, and not at the plasma membrane, where Raf-1 activation occurs. We are currently examining the role of Pak3 in Raf-1 activation and S338 phosphorylation in cells stimulated with growth factors. We are also attempting to identify the kinases which phosphorylate Raf-1 on S338 under physiological conditions.

H-, K- and N-Ras inhibit myeloid leukemia cell proliferation by a $p21^{WAF1}$-dependent mechanism

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Mutated *ras* genes are frequently found in human cancer. However, it has been shown that oncogenic *ras* causes an antiproliferative effect in primary cells, through pathways involving p53 and the cell cycle inhibitors $p16^{INK4a}$ and $p19^{ARF}$. In this work we have analyzed the effect of the ectopic expression of the three mammalian *ras* genes on the proliferation of K562 leukemia cells, which derive from a chronic myeloid leukemia and are deficient for p53, $p16^{INK4a}$ and $p19^{ARF}$ genes. We have first subcloned oncogenic and wild-type *ras* genes in the same expression vector (pCEFL) to get more reliable comparison of their biological activities. We have found that oncogenic H-, K- and N-*ras* inhibit the clonogenic growth of K562 cells, being H-*ras* the most potent inhibitor. The overexpression of the wild type *ras* genes also inhibited proliferation, but to a lesser extent than their oncogenic versions. This effect is not related to apoptosis and co-expression of Bcl-2 did not abrogate the Ras-mediated growth inhibition. We generated K562 sublines with inducible expression of H-RasV12. Induction of H-*ras*V12 retards growth and this effect was accompanied with an increase of $p21^{WAF1}$ mRNA and protein levels. Using a luciferase-reporter gene we found that $p21^{WAF1}$ promoter is potently activated by oncogenic *ras* and less pronounced by wild type *ras*. This transactivation was independent from p53. Moreover, inhibition of $p21^{WAF1}$ expression by an antisense construct partially rescued the inhibition of clonogenic growth mediated by oncogenic H-*ras*. Altogether, these results indicate that the antiproliferative effect of *ras* in myeloid leukemia cells is associated to the induction of $p21^{WAF1}$ expression and suggest the existence of $p19^{ARF}$ and $p16^{INK4a}$-independent pathways that may account for *ras*-mediated growth inhibition. Finally, it is of note that K562 derive from a human chronic myeloid leukemia, and thus our results are consistent with the absence of *ras* mutations in these leukemias.

WIP, the WASP-interacting protein mediates Cdc42/N-WASP filopodia formation.

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Wistkott-Aldrich Syndrome (WAS) is an X-linked immunodeficiency caused by mutations that affect the WAS protein (WASP). Although the physiological function of WAS remains unknown, **WASP** has been implicated in signal transduction and in cytoskeletal control on hematopoietic cells.

We cloned a novel WASP Interacting Protein, WIP (Ramesh et al. 1997, PNAS 94: 14671-6). WIP is a proline rich protein that shows homology to the yeast protein **verprolin,** which is implicated in the organization of the yeast actin cytoskeleton. We have recently demonstrated that WIP and verprolin are functional homologues (Vaduva et al 1999, JBC 274(24): 17103-8). WIP to suppreses the defects in cytoskeletal organization and endocytosis observed in verprolin deficient yeast cells.

N-WASP is a WASP homologue that is ubiquitously expressed (Miki et al. 1996, EMBO J. 15: 5326-35). Induction of **filopodia** by bradykinin is dependent on activation of the small **GTPase Cdc42** and its binding to N-WASP (Miki et al 1998, Nature 391: 93-96). Moreover, N-WASP links Cdc42 to the Arp2/3 complex mediated actin polymerization (Rohatgi et al 1999, Cell 97: 221-231).

Given the homology between WASP and N-WASP we investigated whether N-WASP is also a binding partner of WIP. We show that the WASP binding protein, **WIP, interacts with N-WASP**, binds actin and increases cellular-actin content. Over-expression of WIP in NIH 3T3 fibroblasts causes filopodia formation. This was dependent on WIP binding to actin and on N-WASP. Microinjection of anti-WIP antibody inhibited filopodia induction by bradykinin, an activate Cdc42 mutant (Cdc42v12) and N-WASP. Cdc42, N-WASP and WIP were shown to form a ternary complex. Bradykinin stimulation caused translocation of WIP to the cortical cytoskeleton at areas of filopodia formation. These results suggest that WIP is critical for bradykinin mediated Cdc42/N-WASP dependent filopodia formation.

Ras and Rho Influence on p21$^{WAF1/CIP1}$ Protein Stability

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Ras activates signaling pathways that lead to cell cycle progression, however, high intensity Ras signaling may lead to cell cycle arrest via the induction of the cyclin dependent kinase inhibitor p21$^{WAF1/CIP1}$ (p21). Ras activates, while Rho suppresses, transcription from the p21 promoter, however, little is known about the effect of Ras and Rho on p21 protein stability. When NIH 3T3 cells were transiently transfected with a plasmid encoding p21 driven by a CMV promoter, treatment with the proteasome inhibitors lactacystin (LC) or acetyl-leu-leu-norleucinal (ALLN) increased steady-state p21 protein levels relative to untreated cells. Co-transfection with oncogenic V12 H-Ras increased p21 levels in untreated cells but LC did not elevate p21 further. In addition, p21 protein levels did not differ between untreated and LC or ALLN treated V12 H-Ras transformed Swiss 3T3 cells. These results indicate that proteasome function normally significantly influences steady-state p21 protein levels but Ras transformation reduces the effect of this degradative pathway. Pulse-chase metabolic labeling experiments in NIH 3T3 cells showed that Ras increased the half-life of p21 by~50% and that this effect was blocked by the MEK inhibitor U0126 and the PI-3K inhibitor LY-294002. The Ras stabilization of p21 was mimicked by co-transfection of p21 with cyclin D1, a protein previously shown to be up-regulated following Ras activation.

Co-transfection of p21 with the Rho inhibitor C3 ADP-ribosyl-transferase doubled its half-life while this effect was reversed by a C3-insensitive mutant form of Rho. Significant stabilization of p21 was also observed following treatment of cells with the actin-disrupting drugs Cytochalasin D or Latrunculin B. These results suggest that maintenance of the actin cytoskeleton by Rho is essential for normal turnover of p21 protein.

Further characterization of p21 degradation will help us understand how Ras and Rho function contribute to cell cycle progression and may lead to the development of novel anti-cancer therapeutic agents.

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Schizosaccharomyces pombe rho1p and rho2p GTPases regulate the cell wall biosynthesis through the PKC homologs, pck1p and pck2p.

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Schizosaccharomyces pombe rho1$^+$ is required for maintenance of cell integrity and polarization of the actin cytoskeleton (Arellano *et al.*, 1996; Arellano *et al.*, 1997). However, no other effector besides the (1,3)β-D-glucan synthase enzyme has been identified in *S. pombe*. We have further investigated if *rho1*$^+$ or *rho2*$^+$ signalling could be mediated by the two protein kinase C homologues, pck1p and pck2p. We show in this study that both kinases interact with rho1p and rho2p only when bound to GTP, as most GTPase effectors do. Interestingly, the interaction was mapped in a different part of the proteins than in *Saccharomyces cerevisiae* Pkc1p (Nonaka et al., 1995). Thus, active rho proteins bind to the amino terminal region of the pcks where two HR1 motifs are located, and binding to the GTPase dramatically stabilizes the kinases. We also show that *Δpck1* and *Δpck2* strains present a number of defects related to the cell wall, indicating that this structure might be co-ordinately regulated by both kinases. Detailed biochemical analysis suggests that pck2p is more important in the regulation of the (1-3)β-D-glucan and (1-3)α-D-glucan synthesis. Thus, overexpression of *pck2*$^+$, but not *pck1*$^+$, caused a general increase in cell wall biosynthesis and rho1-regulated (1-3)β-D-glucan synthase activity was considerably augmented. On the other hand, genetic evidence indicates that both *pck1*$^+$ and *pck2*$^+$ interact with *cps1*$^+$ and *gls2*$^+$, two genes similar to *S. cerevisiae FKS1* and *FKS2* that encode membrane subunits of the (1-3)β-D-glucan synthase. It seems therefore that *rho1*$^+$ directly activates the (1-3)β-D-glucan synthase and also regulates (1-3)β-D-glucan biosynthesis mainly through *pck2*$^+$ but also through *pck1*$^+$. On the contrary, *rho2*$^+$ does not affect (1-3)β-D-glucan biosynthesis (Hirata *et al.*, 1998) but rho2p overproduction results in an increase of the cell wall (1-3)α-D-glucan and is lethal in wild type or *Δpck1* cells but not in *Δpck2* suggesting that pck2p is the rho2p main effector.

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TRANSCRIPTIONAL INHIBITION OF PRE-PRO-ENDOTHELIN-1 EXPRESSION BY HMG-CoA REDUCTASE INHIBITORS IN VASCULAR ENDOTHELIAL CELLS. ROLE OF GERANYLGERANYLATED PROTEINS

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Endothelial dysfunction, one of the earliest alterations of atherosclerosis, is characterized by an imbalance between endothelial-derived vasoconstrictors and vasodilators. The HMG-CoA reductase inhibitors (statins), widely used as hypocholesterolemic agents, ameliorate endothelial dysfunction with independence of significant reductions in cholesterol levels. Until recently, the physiological basis for this observation remained unclear. We have previously shown that statins are able to inhibit the expression of endothelin-1 (ET-1), which is a powerful vasoconstrictor and mitogenic peptide, in vascular endothelial cells (Hernández-Perera *et al.*, 1998). Here we show that simvastatin inhibits the transcription of the pre-proendothelin-1 gene (ppET-1). This inhibition can be reverted by mevalonate but not by native LDL. Of the intermediates of the isoprenoid pathway, farnesyl pyrophosphate, at concentrations effective to support ras prenylation, did not prevent the effect of simvastatin on ppET-1 expression. In contrast, geranylgeranyl pyrophosphate, did not support ras processing but significantly reverted the inhibition of ppET-1 expression by simvastatin. The inhibitory effect of simvastatin on ppET-1 expression was mimicked by the geranylgeranyltransferase I inhibitor GGTI-286 but not by the farnesyltransferase inhibitor FTI-277. Finally, treatment of endothelial cells with C3 exoenzyme from *Clostridium botulinum* recapitulated the effect of simvastatin. These results suggest that inhibition of RhoA could be involved in the regulation of endothelin-1 expression by simvastatin and provide new information about the role of small GTP-binding proteins in endothelial function.

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REGULATION OF RHO ACTIVITY BY RAS

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Small GTPases control a wide range of cellular processes by switching between inactive GDP-bound and active GTP-bound states. Ras family GTPases regulate growth factor induced signalling events and frequently mutated in human tumours (reviewed 1). Rho family GTPases co-ordinate regulation of the cytoskeleton and motility (reviewed 2). RhoA regulates the formation of stress fibres and focal adhesions. Both Ras and Rho GTPases are able to regulate cell cycle progression; furthermore Ras requires RhoA to promote cell cycle progression (reviewed 3). Transformation by Ras in fibroblast models can be blocked by inhibition of Rho family GTPases or inhibition of ROCK, a RhoA effector kinase (4,5). However, the majority of studies concerning the role of Rho in Ras-transformation have used fibroblast models; whereas human tumours bearing oncogenic Ras mutations are predominantly from epithelial cells. We have examined whether Rho or ROCK are required for cell cycle progression in untransformed and Ras-transformed human epithelial cells and human tumour cell lines. Additionally we have analysed the effects of Rho or ROCK inhibition on the levels of the cell cycle inhibitory molecules p21 and p27.

Previous studies give conflicting indications as to the effect of Ras-transformation on Rho activity; the Rho-dependence of Ras-transformation may indicate that Rho activity is increased by Ras signalling. Whereas, the loss of actin stress fibres and focal adhesions in transformed fibroblasts could indicate that Rho activity is down regulated (4). To address these seemingly contradictory results, we have used a Rho-GTP pulldown assay to directly analyse endogenous Rho-GTP levels in both Swiss 3T3 fibroblast and HB4A epithelial cell models of Ras-transformation. We have used specific inhibitors of the MAP kinase and PI-3-kinase Ras effector pathways to determine how Ras affects Rho-GTP levels. Additionally, we have investigated how activation of the MAP kinase pathway affects Rho activity.

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***Drosophila RhoGAP*, a new component of the RhoGTPase mediated signalling pathways.**

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The RhoGTPases, RhoA, Rac1, Rac2 and Cdc42, mediate a wide spectrum of cellular functions[1]. In *Drosophila*, they are involved in the establishment of epithelial planar polarity (EPP), which is reflected in the orientation of the wing hairs and ommatidia, in embryonic nervous system development and in the localised activation of the JNK pathway required for embryonic dorsal closure. Like other GTPases, activity of Rho-GTPases is determined by the ratio of the GTP/GDP-bound forms present in the cell. This ratio is regulated by the opposing effects of guanine nucleotide exchange factors (GEF), which enhance the exchange of GDP for GTP, and of GTPase-activating proteins (GAP), which increase the intrinsic rate of hydrolysis of bound GTP. We have cloned a new *Drosophila* gene that encodes a putative GAP for Rho-GTPases, the *DRhoGAP* gene. The predicted aminoacid sequence of *DRhoGAP* shows the highest homology with *Drosophila* Rotund, a putative Rac-GAP, and presents high similarity in the GAP domain with Rho, Rac and Cdc42 GAPs from human, yeast and *C. elegans*. The *Rho-GAP* gene is expressed ubiquitously during the initial stages of embryogenesis and restricted after germ band retraction to the nervous system. During larval and pupal stages its expression in the imaginal disc is generalized.

We generated transgenic flies which allow controlled expression of antisense *DRhoGAP* RNA or dominant negative (DN) versions of the DRhoGAP. Overexpression of any of these transgenes causes an embryonic dorsal open phenotype and in the wing, disruption of the polarity of the wing hairs, a multiple wing hair phenotype, loss of wing tissue and development of large cells, excess vein material and appearance of supernumerary sensory organs. These phenotypes resemble those caused by overexpression of DN forms of the RhoGTPases or by activation of the Ras pathway. These results, and additional genetic interactions, suggest the involvement of *DRhoGAP* in the signalling pathways controlled by Rho and RasGTPases.

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FOCUS FORMATION BY INDUCED ONCOGENIC RAS IN NIH3T3 CELLS

Ras transformation of immortalized murine fibroblasts leads to focus formation, anchorage independent growth and tumorigenesis. This can be effected by overexpression of the wild type, guanine nucleotide-binding Rasp21 protein, or by low levels of constitutively active forms. A range of oncogenic mutants that are defective in either GTPase activity or in nucleotide binding, express increased concentration of the active, GTP-bound form of Ras, which in turn results in increased signalling through the Ras signal transduction pathway. Such mutants are found in a significant fraction of human tumors, and Ras transformation of cultured cells constitutes a model for analysis of the molecular events responsible for the phenotypes of cancer cells.

To have a system in which the level of Ras activity can be conditionally regulated, we have developed several inducible Ras cell lines in NIH3T3 cells, using both the Lac Repressor as well as the Tet Repressor, in which the expression of oncogenic Ras can be controlled.

We have analyzed growth in these cell lines under induced and uninduced conditions, as well as in constitutively transformed cell lines and non-transformed, vector transfected control cells. The Ras cell lines formed foci when plated together with parental NIH3T3 cells; for the inducible cell lines, focus formation was low or non-existent under repressed conditions.

The kinetics of the Ras protein induction reveal elevated levels of Ras 10 - 14 hrs after induction, and analysis of expression of cyclin D1 and the Cdk inhibitor p27 showed the expected high D1 expression in Ras cells at high densities. p27Kip protein, however, accumulated to the same level in Ras and normal cells as the cells grew dense.

We found that the growth rates of Ras transformed cells were identical with the non-transformed control until the cell densities that led to contact inhibition in the non-transformed cells were reached. The Ras cells escaped contact inhibition, although their growth was significantly affected at high cell densities as well. In response to serum deprivation, both Ras transformed and normal cells continued exponential growth at equal rates until a cell density which was dependent on the serum concentration. Induction of oncogenic Ras expression in contact inhibited cells led to a resumption of cell growth; conversely, repression of Ras expression in cells growing at densities where normal cells do not, led to a reduction in growth rate and a final cell density at a level intermediate between that of fully transformed and non-transformed cells. Thus, Ras transformation was reversible.

Our results suggest that the Ras phenotype is not attained primarily through a modulation of the components of the cell cycle, since cell doubling times were unaffected by Ras transformation under both high and low serum conditions. In addition, we find that both non-transformed cells as well as the transformed Ras cells were dependent on serum for growth.

Dissecting ras inhibition from growth arrest: ras activity is not necessary for regulation of cyclin D1 or $p27^{kip1}$ in Rb-negative cells

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The identification of cell cycle targets of the protooncogene product $p21^{ras}$, has been the subject of intense research in the past years. The most common strategy to identify these targets is to inhibit Ras activity with the dominant negative mutant rasN17. Using this kind of approach, several possible cell cycle targets of ras have been identified, including the important cell cycle proteins cyclin D1 and $p27^{kip1}$. A caveat to this kind of experiments however, is that cells arrest their proliferation when the activity of Ras is inhibited, and it is difficult to know whether the effects seen are due to the inhibition of Ras, or are secondary to the growth arrest cells undergo under these circumstances. To avoid this, we have used immortalized Rb-negative fibroblasts as a model system to study cell cycle events dependent on Ras. These cells do not growth arrest when their ras activity is inhibited, even though the regulation of their cycle is well conserved. Thus, inhibition of Ras activity in these cells can help identify cell cycle events that depend directly from Ras.

We have generated stable cell lines expressing rasN17 from Rb-negative fibroblasts. These lines show increased Ras immunoreactivity as assayed by western blot. They also show a greatly impaired MAP Kinase activity in proliferating cells, what indicates Ras activity is inhibited in these cells. Despite this, rasN17 lines proliferate normally. DNA analysis by flow cytometry shows these cells distribute in all phases of the cell cycle, and there are not consistent differences in DNA content profile between control cells and cells expressing rasN17. rasN17 cells can enter S phase on restimulation after serum deprivation, and the length of their G1 phase is similar to control Rb-negative cells. Surprisingly, rasN17 cells do not show inhibition of cyclin D1 expression, both at the level of protein and mRNA. Cyclin D2 and D3 are also expressed at levels similar to control cells. Furthermore, $p27^{kip1}$, a protein whose downregulation at the end of G1 has been proposed to be under the control of ras, behaves in rasN17 cells indistinguishably to control cells. We are at present studying other cell cycle-regulated events in rasN17 cells. A detailed evaluation of cell cycle regulation in these cells should help identify cell cycle targets of ras.

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