Instituto Juan March
de Estudios e Investigaciones

144 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

Exchange Factors

Organized by

X. R. Bustelo, J. S. Gutkind and P. Crespo

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INDEX

PAGE

Introduction

Piero Crespo and Xosé R. Bustelo

Small GTP-binding proteins of the Ras superfamily operate as key molecular switches in signal transduction routes that convey stimuli received in cell surface receptors to the interior of the cell. Today, it is very clear that these proteins play essential roles in the regulation of basic cellular processes such as proliferation, differentiation and apoptosis. Thus, their malfunction can lead to extreme pathologic conditions like cancer.

The hallmark of Ras GTPases function is the transit between an inactive state, in which they are bound to guanosine diphosphate (GDP), and an active state bound to guanosine triphosphates (GTP). This cycle is a strictly regulated process, in which Guanine nucleotide Exchange Factors (GEFs) bring about the activation of the GTPases by catalyzing the nucleotide exchange process. Logic dictates, that a fine-tuning of such an essential system requires that GEFs themselves must also be subject to tight regulatory processes. Indeed, it looks as if evolution has taken this matter very seriously and has provided GEFs with a wide array of regulatory domains. The role of many of these domains, in most cases, is still largely unveiled. Even a domain like the Dbl-Homology (DH) domain characteristic of Rho family GEFs, that orthodoxy has long regarded as a *bona fide* catalytic domain, still inspires doubts about whether it also stores other, hitherto unknown, functions. A ripe field in which much efforts are being invested.

Focusing on GEFs for Ras, we have learned that some like Ras-GRF1 and the Cal-DAG family are not only regulated by stimuli that elevate intracellular calcium levels or generate diacylglycerol. These GEFs are also subject to a tight control that is dependent on the cellular location or membrane compartment in which they are acting upon their cognate Ras GTPases. A similar situation is evident on some Rho GEFs like GEF-H1 whose activity is dependent on its interaction with microtubules. Location: a new aspect of GEF regulation is beginning to unfold.

Regardless of whether the regulation of GEFs is achieved through reversible modifications, like phosphorylation or lipidic additions, or by the interaction with other regulatory proteins, GEFs are subject to profound structural changes. In this field, the resolution of the crystal structures of GEFs catalytic domains in complex with their cognate GTPases and of several GEFs regulatory domains, have provided a large body of invaluable information that has enabled profound advances in our understanding of how GEFs work. These techniques, that are under a process of continuous refinement, are providing an essential tool for probing GEFs functions.

The unquestionable importance of GEFs from the biochemical point of view is clearly mirrored when looking at biological readouts. Right from the bottom of the evolution ladder, some bacteria have developed successful weapons on their struggle against the defensive arsenals of their eukaryotic hosts. Proteins that mimic GEF functions and different toxins that can either inhibit or activate GTPases, enable bacteria to orchestrate the cellular machinery to work for their own benefit. We have witnessed how GEFs begin to display their fundamental functional and structural characteristics in yeast and how these evolve in mammalians, to become key players in processes ranging from the regulation of the immune response to the control of cell proliferation. And how when GEF function is either missing or gone astray, the consequences are dire.

All in a nutshell, this workshop has gathered most of the leading scientist on the field of GEFs. Intending to provide an insight on the latest trends on the subject, approached from different perspectives. We have been enlightened by pouring new data. We have been frightened by the speed with which the field evolves and new technologies are absorbed and rendered essential to keep on investigating. And, above all, we have been humbled by how much there is still to learn in this fascinating area.

Piero Crespo and Xosé R. Bustelo

Session 1: Structural basis of GEFs function
Chair: Dafna Bar-Sagi

Kinetic and structural studies on GEF interactions

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Gaunine nucleotide binding proteins (GNBPs) cycle between a GDP-bound inactive state and a GTP-bound active state. The switch-ON reaction involves the exchange of tightly bound GDP against GTP, while the switch-OFF mechanism involves the enzymatic cleavage of GTP to GDP. The first reaction is catalysed by guanine nucleotide exchange factors GEFs, while the second is activated by GTPase-activating proteins GAPs.

The GEF reaction is a multistep reaction with many intermediates, whereby a binary GNBP-nucleotide complex is converted to a trimeric state of GNBP with bound nucleotide and GEF, which relaxes into a binary GNBP-GEF complex. The reversal of this reaction, under appropriate conditions and with excess GTP leads to an accumulation of GNBP-GTP. Mechanistic details will be presented.

Epac is a guanine nucleotide exchange factor specific for Rap, which is inactive in the ground state and becomes activated by the addition of the second messenger cAMP. Intrasteric inhibition is achieved by the interaction of the N-terminal regulatory domain(s) with the C-terminal catalytic domain, which is relieved by cAMP. Structural and mechanistic investigations of activation will be presented.

Mechanistic studies of Rho GTPase activation by guanine nucleotide exchange factors

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The signaling pathways mediated by Rho family GTPases have been implicated in many fundamental aspects of cell biology. The specificity of Rho GTPase pathways is achieved in part by selective interaction between the immediate upstream Dbl family guanine nucleotide exchange factors (GEFs) and Rho GTPase substrates. Based on the structural data of Tiam1-Rac1 complex, we have previously identified a groove formed by the switch I, switch II, and β2/β3 regions of Rac1 as the key area involved in GEF-specification, and predicted that reagents blocking Rac recognition by GEFs in this area might be developed into Rac activation-specific inhibitors. Here we report the identification and characterization of a first generation inhibitor targeting at Rac1 activation by specific interference of the GEF-Rac interaction. We have discovered a small chemical compound that is highly soluble in aqueous phase, is membrane permeable and fits well into the surface cavity of Rac1 that is critical for GEF recognition in a simulated docking model. In vitro the compound effectively inhibited Rac1 binding and activation by Trio in a dose dependent manner (IC50 at ~40 μM) but did not interfere with Cdc42 activation by intersectin. In NIH 3T3 cells, it potently blocked serum or PDGF induced Rac1 activation and inhibited PDGF stimulated lamellipodia formation at 50 μM without affecting the activity of endogenous Cdc42. When applied to human prostate cancer cells, it significantly slowed the tumor cell proliferation and inhibited the cell invasion into matrigel matrix. This compound may therefore act as a Rac activation-specific inhibitor and could be employed to study the role of Rac in various cellular systems.

The mechanistic roles of GEFs in small G-protein activation reaction have been thought to stimulate GDP dissociation and to stabilize a nucleotide-free G-protein intermediate. In a separate study we show that Trio activates Rac1 by facilitating GTP binding to Rac1. The GEF reaction appears to undergo a novel two nucleotide-one G-protein intermediate, GTP-Rac-GDP. The simultaneous binding of GDP and GTP in the transition state may involve one cryptic site of the G-protein under GEF induction, with one part contributing to the recognition of the β/γ phosphates of incoming GTP and another part to the binding of the guanine base of the leaving GDP. We propose that activation of Rho GTPases by GEFs is likely through direct displacement of bound GDP by GTP rather than sequential GDP dissociation and GTP association.

Structure-function studies of the Ras exchanger Sos

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Ras proteins are key intermediates in signal transduction 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 mechanochemical basis for the guanine nucleotide exchange reaction catalyzed by Sos as revealed by structure-function studies will be discussed (1,2). In particular, recent structural and biochemical evidence indicating a positive feedback regulation of Sos activity by Ras-GTP will be presented. In addition, new insights into the mechanisms by which the exchange activity of Sos is controlled by intramolecular interactions will be described (3).

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The Vav/Rac route positively regulates Ras signaling by direct activation of the Ras guanosine nucleotide exchange factor CalDAG/Ras GRP

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In order to generate coherent biological responses to extracellular stimuli, cells have established synergistic and antagonistic cross-talks between pathways with similar or opposing functions, respectively. Two routes cooperating in the generation of mitogenic and cytoskeletal functions are those induced by the GTPases Rho/Rac and Ras. It has been reported that these two pathways interact at different levels, including the activation of Raf and MEK1 by the Rac downstream effector PAK and the stimulation of Rac1-specific guanosine nucleotide exchange factors by phosphatidylinositol 3-kinase, a known Ras effector (1). In this report, we describe an entirely novel cross-talk mechanism between these two pathways based on the direct stimulation of CalDAG/Ras GRP proteins by Vav family members and Rac1. This cross-talk is dependent on the integrity of the phospholipase C-γ pathway, since specific mutations eliminating the activity of this route impair the activation of CalDAG/Ras GRP by Vav. The activation of CalDAG/Ras GRP by the Vav/Rac pathway involves an additional step consisting of the active translocation of CalDAG/Ras GRP to peripheral cellular structures enriched in F-actin. This cross-talk mechanism is crucial for optimal Ras activation in lymphoid cells, since this GTPase cannot become activated in the absence of Vav proteins. Taken together, these results indicate that a relay mechanism exists in lymphoid cells assuring robust signaling responses of the Rac/Rho and Ras pathways upon the engagement of antigen receptors.

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Rate of basal Ras nucleotide exchange conditions outcome in agonist-induced Ras activation

Ignacio Rubio, Knut Rennert, Ute Wittig, Reinhard Wetzker

Extracellular agonists promote Ras activation via changes in the activity of guanine nucleotide exchange factors (GEFs) and/or GTP hydrolase activating proteins (GAPs). These agonist-dependent effects superimpose on constitutively operating GEF and GAP systems that sustain basal Ras-GDP/GTP cycling in resting cells. A number of studies have illustrated markedly divergent rates of constitutive nucleotide turnover on Ras among different cell types. It is conceivable that dynamic versus low-rate constitutive Ras-GDP/GTP cycling conditions the outcome in agonist-induced Ras activation. To address this issue we have employed a permeabilization strategy to assess Ras nucleotide uptake rates and Ras-GDP/GTP levels at pre-steady state nucleotide turnover conditions. We find that inhibition of various signalling activities compromises basal nucleotide turnover on Ras, elevating the threshold for agonist-induced nucleotide exchange. In particular our data implicate the EGF receptor and PI3K in the regulation of basal Ras nucleotde exchange. As a consequence EGFR or PI3K inhibition impede agonist-driven net Ras-GTP accumulation in spite of unaffected agonist coupling to GEFs. These findings establish the existence of a permissive level of Ras activation control exerted through regulation of basal Ras nucleotide exchange.

Mechanism of domain closure of Sec7 domains and role in BFA-sensitivity

Louis Renault, Petya Christova, Bernard Guibert, Sebastiano Pasqualato and Jacqueline Cherfils

Activation of small G proteins of the Arf family is initiated by guanine nucleotide exchange factors whose catalytic Sec7 domain stimulates the dissociation of the tightly bound GDP nucleotide. The exchange reaction involves distinct sequential steps that can be trapped by the non-competitive inhibitor Brefeldin A, by mutation of an invariant catalytic glutamate, or by removal of guanine nucleotides. Arf-GDP retains most characteristics of its GDP-bound form at the initial low-affinity Arf-GDP-Sec7 step. It then undergoes large conformational changes towards its GTP-bound form at the next step, and eventually dissociates GDP to form a nucleotide-free high-affinity Arf-Sec7 complex at the last step.

Thus, Arf proteins evolve through different conformations that must be accommodated by Sec7 domains in the course of the reaction.

Here the contribution of the flexibility of Sec7 domains to the exchange reaction was investigated with the crystal structure of the unbound Sec7 domain of yeast Gea2. Comparison with Gea2 in complex with nucleotide-free Arf117 (1) reveals that Arf induces closure of the two subdomains that form the sides of its active site. Several residues that determine sensitivity to Brefeldin A are involved in interdomain and local movements, pointing to the importance of the flexibility of Sec7 domains for the inhibition mechanism. Altogether, this suggests a model for the initial steps of the exchange reaction where Arf docks onto the C-terminal domain of the Sec7 domain before closure of the N-terminal domain positions the catalytic glutamate to complete the reaction.

Session 2: GEFs in lower organisms
Chair: Jorge E. Galán

Bacterial toxins and effectors acting on Rho GTPases

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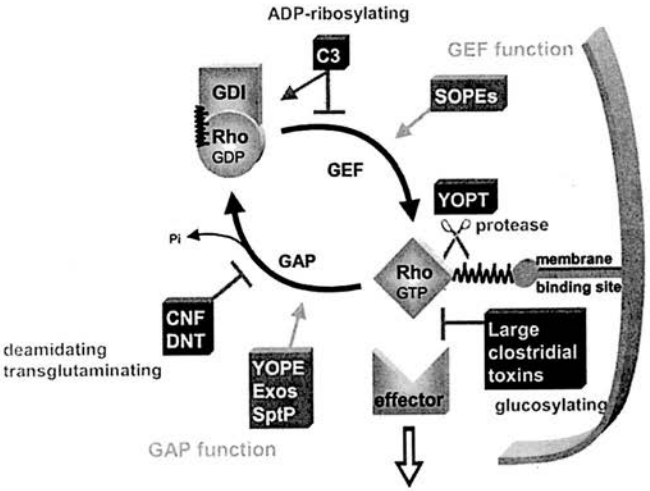
Rho proteins comprise a family of more than 15 low molecular mass GTPases, which function as molecular switches in signal processes, including regulation of the actin cytoskeleton, smooth muscle contraction, transcriptional activation, cell cycle progression and control of acquired and innate immunity. Moreover, the GTPases are the eukaryotic targets of various bacterial protein toxins and effectors, which activate and inactivate the GTPases, respectively.



ADP-ribosylation of Rho is catalyzed by C3-like transferases. The ~25kDa enzymes are produced by C. botulinum, C. limosum, B. cereus and S. aureus. C3 transferases catalyze the ADP-ribosylation of RhoA, B, and C at Asn41. ADP-ribosylation of Asn41 inhibits Rho activation by guanine nucleotide exchange factors (GEFs, e.g., Lbc), alters Rho membrane binding and increases the interaction with the guanine nucleotide dissociation inhibitor GDI, but does not inhibit the interaction of Rho with Rho effectors like Rho kinase. Members of the family of large clostridial cytotoxins, including C. difficile toxins A and B, C. sordellii lethal and hemorrhagic toxins and C. novyi alpha toxin) glucosylate Rho GTPases at Thr37/35 of Rho, Rac and Cdc42 depending on the toxin subtype. Glucosylation inhibits the interaction of Rho GTPases with their effectors. In addition, glucosylation blocks GTP hydrolysis by Rho and its activation by Lbc. Recently, it was shown that the Yersinia effector YopT inactivates Rho GTPases by cleavage of the isoprenylated C terminus, resulting in membrane release of

Rho proteins. Moreover, several bacterial effectors inactivate Rho GTPases by acting in a manner like GTPase–activating proteins (GAPs), including Pseudomonas aeruginosa exoenzyme S, Yersinia YopE and Salmonella SptP.

Rho GTPases are activated by the cytotoxic necrotizing factors CNF1 and 2 from E. coli, which deamidate RhoA at Gln63 (Rac/Cdc42 at Gln61). Deamidation results in inhibition of the GTP-hydrolyzing activity of the Rho proteins, forming constitutively active proteins. The dermonecrotizing toxin DNT from Bordetella causes deamidation or transglutamination at the same site of Rho. Finally, Rho GTPases are activated by bacterial GEFs including Salmonella SOPE proteins.

The various bacterial toxins and effectors are not only important virulence factors in bacterial diseases but also cell-biological and pharmacological tools to study their mammalian targets.

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Subcellular localization of Cdc24p, the Cdc42p-specific GEF in *S. cerevisiae*, is a function of specific targeting and efficient anchoring within a cytoskeletal complex

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S. cerevisiae Cdc24p, a founding member of the Dbl oncogene family of Rho GTPase guanine-nucleotide exchange factors (GEFs), activates Cdc42p-dependent signaling pathways that modulate actin polymerization and polarized growth (5). Cdc24p localizes to the nucleus in haploid yeast cells and to sites of polarized growth, including the incipient bud sites, tips of enlarging buds, and the mother-bud neck region, during the mitotic cell cycle (2, 6, 8). Cdc24p and its substrate Cdc42p co-localize at polarized growth sites and the carboxyl-terminal 289 amino acids of Cdc24p are necessary and sufficient for this localization (2, 6, 8). The phosphoinositide binding sequences within the conserved pleckstrin homology (PH) domain of Cdc24p, which are involved in membrane localization of other Rho GEFs, are not included in these sequences, raising the possibility that other targeting mechanisms may be involved. Two potential calcium-binding C_2 domains are present within these sequences along with a PC domain that interacts with the PB1 domain of the Bem1p scaffold (4, 7), which has been implicated in maintaining, but not targeting, Cdc24p at sites of polarization (1-3). Analysis of *cdc24* truncation and site-specific mutations revealed the presence of a necessary and sufficient 56 amino-acid targeting domain and a separate carboxyl-terminal anchoring domain (Toenjes *et al.*, submitted). Mutant analysis and protein solubilization data indicated that efficient Cdc24p anchoring within a membrane-associated cytoskeletal complex required the Bem1p scaffold protein, the Rsr1p/Bud1p GTPase, and the novel transmembrane protein YGR221Cp. These data are consistent with Rho GEF localization being a function of both membrane-specific targeting and subsequent anchoring within multi-protein cytoskeletal complexes. Given the highly conserved roles of GEFs in Cdc42p signaling pathways, it is likely that similar targeting and anchoring mechanisms exist for Rho GEFs in other eukaryotes.

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Mimicry as a strategy to modulate cellular functions by a bacterial pathogen

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The bacterial pathogen Salmonella enterica has evolved a very complex functional interphase with its host, the product of the work of co-evolutionary forces operating over millions of years. Central to this interphase is the work of a specialized bacterial organelle, the type III secretion system, which delivers a battery of bacterial proteins into the host cell. These proteins have the capacity to modulate a variety of cellular processes ranging from actin cytoskeleton rearrangements and nuclear responses to macropinocytosis and programmed cell death. A theme emerging from structural and functional studies of the Salmonella/host interactions is one of mimicry as a strategy to modulate cellular functions. These bacteria utilize proteins that faithfully mimic, structurally and functionally, the activities of host cell protein to modulate a variety of responses. Two bacterial proteins, SopE and SopE2, work as exchange factors for the Rho-family GTPases, Cdc42 and Rac. Remarkably, although through different chemistry, the conformational changes induced by these bacterial proteins in the host GTPases, which result in nucleotide exchange, are virtually identical to those induced by Dbl-like GEFs. In a remarkable Ying and Yang, another bacterial protein, SptP, mimics host GTPase-activating proteins (GAPs) to reverse the bacterial-induced activation of Cdc42 and Rac. In this case, the Salmonella protein uses the same chemistry as host GAPs (i. e. insertion of a key arginine) but utilizing different structural context. These and other examples will be discussed to illustrate the power of mimicry as a central strategy to modulate cellular functions by microbial pathogens.

T cell activation targets RasGRP and DGKalpha to the membrane; role of DGKalpha as a negative modulator of Ras activation

Sanjuan, MA, Pradet-Balade, B., Jones, DR, Martinez-A, C, Garcia-Sanz, JA and Mérida I.

Diacylglycerol kinase (DGK) phosphorylates diacylglycerol (DAG) to phosphatidic acid (PA), attenuating cellular levels of DAG, a lipid with a central role in T cell activation. Nine DGK isoforms, grouped into five subtypes, are found in higher organisms; all contain a conserved C-terminal domain and at least two cysteine-rich motifs of unknown function. Here we studied DGKa regulation, using a transgenic mouse model in which injection of an antigenic peptide activates the majority of peripheral T cells. Analysis of lymph node T cells at different times after injection shows that DGKa, highly expressed in resting T lymphocytes, is subject to complex control at the mRNA and protein levels during *in vivo* T cell activation. Subcellular fractionation of T lymphocytes shortly after *in vivo* engagement of the T cell receptor shows rapid translocation of cytosolic DGKa to the membrane fraction. DGKa translocation to the membrane correlates with rapid translocation of RasGRP, a nucleotide exchange activator for Ras that associates to the membrane through a DAG-binding domain. To demonstrate a causal relationship between DGKa and RasGRP membrane relocation, we studied RasGRP translocation kinetics in a T cell line transiently transfected with constitutive active and transdominant negative DGKa mutants. We show that localization of DGKa near the antigen receptor serves as a negative regulatory signal for Ras activation by modulating RasGRP translocation.

This study is the first demonstration of *in vivo* regulation of DGKa and RasGRP, and provides new insight into the functional role of a member of this family of lipid kinases in the regulation of the immune response.

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Session 3: Mechanisms for GEFs regulation
Chair: Johannes L. Bos

Regulation of Ras and Rho guanine nucleotide exchange factors

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Extracellular stimuli activation of Ras and Rho GTPase is mediated commonly by activation of guanine nucleotide exchange factors (GEFs) that contain CDC25 or Dbl homology (DH) catalytic domains, respectively. Sequences flanking these catalytic domains determine signal-specific activation of each GEF. We evaluated the regulation and function of two CDC25 homology domain- and three DH domain-containing GEFs. Using a retrovirus expression library, corresponding to mRNA expressed in a patient-derived acute myeologenous leukemia, we identified a new member of the RasGRP family of GEFs as a transforming protein. We designated this CDC25 homology domain-containing protein RasGRP4 and showed that it functions as phorbol ester-regulated activator of Ras but not Rap. Northern blot analyses determined that RasGRP4 expression was restricted to peripheral blood leukocytes and a survey of human hematopoietic tumor cell lines showed that RasGRP4 expression is distinct from other RasGRPs and was restricted to myeloid leukemia cells. Ongoing studies include the generation of mice deficient for RasGRP4 to evaluate its role in myeloid cell signaling and oncogenesis. Phospholipase C epsilon (PLCε) also contains a CDC25 homology domain, but conflicting observations have been reported concerning its GTPase specificity. We determined that it is an activator of Rap but not Ras. The lipase activity of PLCε is stimulated by activated Ras and G alpha 12/13 and we have evaluated whether the Rap GEF activity is regulated by activated GTPases or by activated PI 3-kinase via a PH domain C-terminal to the CDC25 homology domain.

We have evaluated the role of the invariant PH domain immediately adjacent to all DH domains, and other flanking sequences, in regulating the activities of three DH domain-containing proteins identified initially as transforming proteins (Dbs, Ect2, and Tim). We showed previously that deletion of the PH domain abolishes the transforming activity of Dbs and that a plasma membrane targeting sequence fully restored Dbs membrane association but only partially restored Dbs transforming activity. This suggested that the PH domain also serves functions beyond membrane targeting. Therefore, we evaluated the contribution of phospholipid binding to the PH domain in regulating Dbs function. We introduced missense mutations into the PH domain that impaired phospholipid binding. The tandem DH/PH domain, but not the isolated DH domain, shows robust GEF activity, demonstrating a critical role of the PH domain for intrinsic DH domain catalytic activity. Surprisingly, missense mutations in the PH domain did not impair DH domain catalytic activity *in vitro*, yet they greatly impaired catalytic activity *in vivo* (RhoA and Cdc42 activation) as well as transforming activity in NIH 3T3 cells. No regulation of DH domain activity by phospholipids was found *in vitro* or by PI 3-kinase activation *in vivo*. However, Dbs subcellular localization was altered by these PH domain mutations, suggesting that phospholipid binding to the PH domain facilitated membrane association of Dbs.

We noted previously that Ect2 C-terminal sequences adjacent to the PH domain were required for potent Ect2 transforming activity. However, the lack of any known protein domains or motifs in the C-terminus provided no clues to its role in regulating Ect2 function. We found that DH/PH/C and DH/PH fragments of Ect2 displayed equivalent stability or subcellular location. However, whereas DH/PH caused activation of RhoA alone, the DH/PH/C fragment activated RhoA, as well as Rac1 and Cdc42, *in vivo*. Furthermore, Ect2 DH/PH/C caused changes in actin organization distinct from that caused by activation of RhoA alone. We suggest that the C-terminal sequences alter the GTPase specificity of Ect2 *in vivo*, which in turn may contribute to enhanced transforming activity. Other Ect2 studies include determination of the critical role for this Dbl family protein in mouse development.

Finally, we evaluated the regulatory role of sequences flanking the DH/PH domains of Tim. N-terminal deletion of sequences upstream of the DH domain, that contains a putative SH3 domain PXXP binding motif, created a constitutively activated and transforming variant. Since the C-terminus of Tim contains an SH3 domain, we evaluated a model where the SH3 domain formed an association with the N-terminal PXXP motif to inhibit Tim DH domain catalytic activity. However, deletion of the SH3 domain in the C-terminus did not activate Tim transforming activity and when deleted from N-terminal deletion activated Tim, abolished transforming activity. Finally, the SH3 domain did not recognize the N-terminal PXXP motif, indicating that it was not involved in an intramolecular regulatory mechanism of Tim DH domain function. The recognition motif of the SH3 domain and possible binding partners are currently being determined.

Membrane microlocalization dictates specificities on the activation of H-Ras, K-Ras and N-Ras and the function of the exchange factor Ras-GRF 1/2

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Ras GTPases include the isoforms H-Ras, K-Ras and N-Ras. Despite their great biochemical and biological similarities, evidence is mounting suggesting that Ras proteins may not be functionally redundant. A widespread strategy for studying small GTPases is the utilization of dominant inhibitory mutants that specifically block the activation of their respective wild-type proteins. As such, H-Ras N17 has proved to be extremely valuable as a tool to probe Ras functions. However, a comparative study on the inhibitory specificities of H-, K- and N-Ras N17 mutants has not been approached thus far. Herein, we demonstrate that H-, K- and N-Ras N17 mutants exhibit markedly distinct inhibitory effects towards H-, K-, and N-Ras. H-Ras N17 can effectively inhibit the activation of all three isoforms. K-Ras N17 completely blocks the activation of K-Ras and is only slightly inhibitory on H-Ras. And N-Ras N17 can mainly inhibit N-Ras activation. In light of the recent data on the compartmentalization of H-Ras and K-Ras in the plasma membrane, here we present for the first time a description of N-Ras cellular microlocalization. Overall, our results on Ras N17 mutants specificities exhibit a marked correlation with the localization of the Ras isoforms to distinct membrane microdomains.

In this same context, we also present evidence that the Ras exchange factors Ras-GRF 1-2 and SOS differentially activate H-Ras in a fashion dependent on the subcellular compartmentalization. We show that Ras-GRF1/2 and SOS colocalize with H-Ras in the plasma membrane and the endoplasmic reticulum but not in the golgi. By specifically tethering H-Ras to these distinct locations we can monitor H-Ras activation by the different GEFs. By this method, we show that the DH and IQ domains of Ras-GRF 1/2 play an important role in regulating the activation of H-Ras in the different locations. Likewise, the inhibitory effects that Cdc42 displays on the activation of Ras by Ras-GRF, that we have previously reported, are also dependent on microlocalization. Overall, our results point to the subcellular compartmentaliztion of H-Ras as a key determinant of its activation and subsequent functions.

The RGS domain of PDZ-RhoGEF and LARG links $G\alpha_{12/13}$ and their coupled receptors to Rho, whereas their PDZ-domain mediate Rho activation by Plexin B, an axon guiding receptor for semaphorins

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The Rho family of small GTP-binding proteins, including Rho, Rac and Cdc42, regulate the organization of the actin-based cytoskeleton and initiate signaling pathways controlling gene expression. The activity of these small GTP-binding proteins is tightly regulated, and the conversion of their inactive GDP-bound form to active GTP-bound species is promoted by guanine nucleotide-exchange factors (GEFs). Recent work in our laboratory and others led to the discovery that a new family of GEFs for Rho, including PDZ-RhoGEF, LARG and p115RhoGEF can provide a direct link between $G\alpha_{12}$ and $G\alpha_{13}$, and their coupled cell surface receptors to Rho. Of interest, these closely related RhoGEFs exhibit an area of homology to regulators of G protein signaling (RGSs), and recent evidence from our lab and others suggests that this RGS domain provides a structural feature by which G proteins of the $G\alpha_{12}$ family can bind and activate these RhoGEFs, thereby stimulating Rho.

Although these GEFs share many structural domains, PDZ-RhoGEF and LARG are distinctive in that they exhibit a PDZ domain, a modular protein interaction domain that binds to consensus motif (S/TXV) in the carboxyl-terminus of partner proteins or, alternatively, to other PDZ domains. As the precise nature of the GEFs by which most cell surface receptors promote Rho activation is still largely unknown, we hypothesized that PDZ-RhoGEF and LARG may utilize their PDZ domain to interact with novel signaling molecules, thereby leading to Rho activation in response to extracellular stimuli in addition to those acting on G proteins. Using a yeast-two hybrid screen using the PDZ domain of PDZ-RhoGEF as bait, we cloned Plexin B2, a member of a newly discovered family of transmembrane receptors for semaphorins, which has been recently shown to provide attractive and repulsive cues necessary for axon guidance. This interaction was confirmed in eukaryotic cells, and found that the PDZ binding motif of Plexin B1, B2, and B3 and the PDZ domain of PDZ-RhoGEF and LARG were required for this interaction. Furthermore, using an *in vivo* Rho guanine nucleotide exchange assay, we observed that Plexins B can indeed stimulate potently endogenous RhoA, and that these responses required PDZ-RhoGEF and LARG. Our findings, together with recently published reports, indicate that Plexins B signal to Rho through their direct interaction with PDZ-RhoGEF and LARG, thus providing a novel molecular mechanism by which Plexins B mediate the repulsive cues initiated by axon-guiding semaphorins. Recent work on the regulation of Rho and RGS-containing RhoGEFs by GPCRs and plexins, as well the functional consequences of genetically deleting some of these RhoGEFs in mice will be presented.

The Rap1 GEF Epac in integrin-mediated cell adhesion

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We recently identified a novel cAMP target, Epac, which is a guanine nucleotide exchange factor for Rap1. By rational drug design we developed a novel cAMP analog that efficiently activates Epac but not protein kinase A (PKA). In various cell lines this Epac-specific cAMP analog was able to induce Rap1 activation, but not the phosphorylation of the PKA substrate CREB. However, interestingly, both in cell lines where cAMP inhibits growth factor-induced ERK activation and in cell lines where cAMP activates ERK, cAMP-E did not affect ERK activity. Moreover, inhibition of PKA as well as Ras, but not Rap1, inhibited cAMP-mediated ERK activation. Thus in contrast to the popular model that Rap1 mediates cAMP-induced modulation of ERK, our results show that cAMP-induced regulation of ERK and activation of the small GTPase Rap1 are independent processes.

Rap1 has also been implicated in the regulation of integrin-mediated cell adhesion. Indeed ectopic expression of an active Rap1 increased integrin-mediated cell adhesion, whereas inhibition of Rap1 by Rap1GAP inhibits integrin-mediated cell adhesion induced by several stimuli. We now show that stimulation of cells with isoproterenol, which activates the Gs-coupled β2-adrenergic receptor induces integrin-mediated cell adhesion to fibronectin. This induction is independent of PKA, but dependent of Rap1. Moreover, cAMP-E also induces integrin-mediated adhesion to fibronectin. These results show that that β2-adrenergic receptors can regulate integrin activation through the activation of Epac and Rap1.

Phosphorylation of the Ras-GRF1 exchange factor at Serine-916 reveals activation of Ras signaling in the prefrontal cortex

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Combinatorial signalling through integrators of multiple signal transduction pathways is critical to biological response. The guanine nucleotide exchange factors are critical control elements for signalling cross-talk. Ras-GRF1, for example, integrates multiple signalling pathways into the activation of Ras and Rac, providing complex functional regulation. The ability of Ras-GRF1 to activate Ras is increased by multiple serine/threonine phosphorylation events that occur in response to stimulation of G protein-coupled receptors (Mattingly & Macara, 1996), and is also regulated by calcium/calmodulin (Farnsworth et al., 1995).

Ras-GRF1, when it is tyrosine phosphorylated by Src, can also act as an exchange factor for Rac (Kiyono et al., 2000). Through CNBr digestion, 2-D tryptic mapping and MALDI/MS, we have now identified 4 of the residues at which muscarinic receptor stimulation increases Ras-GRF1 phosphorylation in intact cells. Phosphorylation of Serine-916, which is required for full activation of the Ras exchange factor (Mattingly, 1999), we now show to be a regulated phosphorylation event that can be induced by multiple signalling pathways in transfected COS-7 and PC12 cell systems.

Furthermore, activation of PKA increases phosphorylation of endogenous Ras-GRF1 at the equivalent residue (Serine-898) in rat cortical brain slices. Indirect confocal immunofluorescence using a novel antibody that selectively recognizes the phospho916(898)-form of Ras-GRF1 demonstrates that Ras-GRF1 is activated in the dendrites of rat prefrontal cortical neurones. These results therefore provide further mechanistic understanding of the control of the Ras-GRF1 exchange factor. (Supported by NIH RO1 CA-81150).

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Regulation of GEF-H1 by microtubule binding and phosphorylation

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The molecular mechanisms linking microtubule dynamics to the actin cytoskeleton via Rho GTPases have not been identified. We show that the Rho guanine nucleotide exchange factor GEF-H1 is regulated by interaction with microtubules. Thus, microtubule binding-deficient forms of GEF-H1 exhibit higher activity than microtubule–bound forms and induce Rho-dependent changes in cell morphology and actin organization. Directed targeting of microtubule binding-deficient GEF-H1 to microtubules results in suppression of GEF-H1 exchange activity. Microtubule depolymerization induces changes in cell morphology and gene expression similar to those caused by expression of active forms of GEF-H1 and these effects are inhibited by dominant-negative versions of GEF-H1. Thus, GEF-H1 links changes in microtubule integrity to Rho-dependent regulation of the actin cytoskeleton (Krendel et al, Nature Cell Biology 4: 294-301, 2002).

We have identified a region in the C-terminus of GEF-H1 that is important for suppression of its GEF activity by microtubules. This portion of the protein includes a coiled-coil motif, a proline-rich motif that may interact with SH3 domain-containing proteins, and a potential binding site for 14-3-3 proteins. We show that GEF-H1 is a binding target and substrate for p21-activated kinase (PAK1), an effector of Rac and Cdc42 GTPases, and localize the PAK1 phosphorylation site to the inhibitory C-terminal region of GEF-H1. Phosphorylation of GEF-H1 by PAK1 induces 14-3-3 binding to the exchange factor and relocation of 14-3-3 to microtubules. Phosphorylation of GEF-H1 by PAK may be involved in regulation of GEF-H1 activity and may serve to coordinate Rho, Rac and Cdc42-mediated signaling pathways.

Session 4: Biological aspects of GEFs functions
Chair: John G. Collard

B cell development and antigen receptor signalling

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The development of B cells proceeds through a well-defined set of stages (Meffre et al., 2000) many of which require signal transduction by cell surface antibody, the B cell antigen receptor (BCR). In mature B-lymphocytes, activation mediated through the BCR orchestrates integration of the cell cycle, transcription and differentiation. BCR signalling controls the balance between tolerance and autoimmunity and the selection of high affinity antibody producing variants essential for immunological memory.

Vav proteins are guanine nucleotide exchange factors (GEFs) that activate a subgroup of Rho GTPases (Rac1, Rac2 and RhoG) by promoting exchange of GDP for GTP. Vav proteins are rapidly phosphorylated and activated upon BCR engagement, and provide the major, and perhaps only, link from the BCR to Rho family of GTPases(Bustelo, 2001). By generating mice deficient in Vav-1 and Vav-2 we have identified Vav proteins as functional components of the BCR signal transduction machinery (Doody et al., 2001). Vav proteins regulate the BCR triggered calcium fluxes that have been implicated in the selection events that regulate B cell maturation and proliferation. A study of signal transduction in Vav-deficient B cells indicates Vav proteins regulate Btk activation. The ability of Vav proteins to regulate Btk activity places them at a central position in the "signalosome"(DeFranco, 2001) model as both Btk and PLCγ2 activation requires the function of the Vav proteins.

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Vav1: a key signal transducer from the TCR

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Vav1 is a guanine nucleotide exchange factor (GEF) for Rac1, Rac2 and RhoG small GTPases, which is expressed throughout the haemopoietic system. Uniquely, the GEF activity of Vav1 and its related family members Vav2 and Vav3, is activated by tyrosine phosphorylation. In particular Vav1 is rapidly tyrosine phosphorylated following engagement of the antigen receptors of B and T cells (BCR and TCR respectively). In addition to its GEF activity, catalyzed by the Dbl homology (DH) domain, Vav1 contains a number of other domains characteristic of signal transducing proteins, including a pleckstrin homology (PH), cysteine rich (CR), and SH2 domain and two SH3 domains. All these features suggested that Vav1 may play an important role in transducing signals downstream of the BCR and TCR.

In order to understand the *in vivo* function of Vav1, we generated mice deficient in Vav1 by homologous recombination in embryonic stem (ES) cells. The resulting mice are alive, fertile and grossly normal (1). Analysis of T cell development showed that in the absence of Vav1 there was a partial block in development at the β-selection checkpoint, consistent with a requirement for Vav1 to transduce a signal from the pre-TCR. Furthermore, there was a much more severe block at the next stage of T cell development, the transition from $CD4^+CD8^+$ double positive (DP) to $CD4^+$ or $CD8^+$ single positive (SP) thymocytes. Using TCR transgenics, we showed that this was due to defective positive selection of DP thymocytes, most likely due to a failure to transduce normal TCR signals (1). Despite defective positive selection, a small number of T cells are generated, however their function is not normal, as exemplified by defective TCR-induced proliferation (2). Furthermore these T cells are unable to provide normal help to B cells, resulting in defective immunoglobulin class switching in response to immunization by T-dependent antigens (3).

Analysis of TCR signalling pathways in Vav1-deficient T cells and DP thymocytes showed reduced intracellular calcium flux, ERK and NF-κB activation (4, 5). Furthermore, while the activation of the proximal tyrosine kinases Lck and ZAP-70 was normal, as was the phosphorylation of the adapter proteins SLP-76 and LAT, the activation and phosphorylation of PLCγ1 was defective. This resulted in reduced production of inositol-2,4,5-trisphosphate (IP_3), a second messenger required for intracellular calcium flux. We have gone on to show that Vav1-deficient DP thymocytes also show defective phosphorylation of the Tec-family kinases Itk and Tec, probably as a result of reduced phosphoinositide-3-kinase (PI3K) activation (5). This latter result was unexpected, as PI3K had been reported to be upstream of Vav1. In view of the critical role of Tec kinases in the activation of PLCγ1, it is likely that the defective PLCγ1 activation in Vav1-deficient cells is due to the failure of Tec kinase activation.

Since Vav1 is a GEF for Rac GTPases, and these in turn have been implicated in the regulation of the actin cytoskeleton, it is possible that Vav1 may transduce TCR signals to the actin cytoskeleton. To examine this in detail, we used Vav1-deficient DP thymocytes expressing the F5 TCR, a class I-restricted receptor specific for peptides from influenza

nuclear protein presented by H-2D^b, and examined a number of actin-dependent events. Surprisingly, we find that Vav1 is required for some but not all actin-dependent events (6). We show that in contrast to wild-type DP thymocytes, Vav1-deficient cells are less efficient at forming conjugates with antigen presenting cells loaded with agonist peptide. Furthermore we demonstrate that Vav1 is required for TCR-induced activation of the integrin LFA-1, which is likely to explain the defect in conjugate formation. However once Vav1-deficient cells form a conjugate, the assembly of proteins into an immunological synapse at the conjugate interface is normal. In contrast, thymocyte polarization is defective in the absence of Vav1, as judged by the relocalization of the microtubule organizing centre. These data demonstrate that Vav1 transduces signals to only a subset of cytoskeleton-dependent events at the immunological synapse.

Finally, in order to gain further understanding of how Vav1 transduces signals we have used homologous recombination in ES cells to create mice bearing subtle mutations in the *Vav1* gene (7). In particular we have made mice bearing the R422G mutation in the PH domain which is predicted to abolish binding of the phospholipids PIP_2 and PIP_3. It has been proposed that binding of PIP_2 to the PH domain of Vav1 inhibits its GEF activity, while binding of PIP_3 activates it. Our results do not support the idea that PIP_2 inhibits Vav1, since mice bearing the Vav1^{R422G} allele show decreased rather than increased TCR-induced signalling. The mutation results in a slight impairment of positive selection in the thymus, as well as a reduction in TCR-induced proliferation, calcium flux and ERK and Rac1 activation, though in all cases the phenotype is milder than that of the complete knockout. Most interestingly the effect of this mutation is selective to the CD4$^+$ T cell lineage, while leaving CD8$^+$ T cells unaffected. Since a complete knockout of Vav1 compromises TCR signaling in both lineages, this result suggests that the mechanism by which Vav1 is regulated differs between the two T cell lineages.

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Tiam1-Rac signaling in tumor formation and progression

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Rho-like GTPases control signaling pathways that regulate the actin cytoskeleton as well as gene transcription. Similar to Ras proteins, Rho-like proteins cycle between an active GTP-bound state and the inactive GDP-bound state. The principal regulators are the guanine nucleotide exchange factors (GEFs) and the GTPase activating proteins (GAPs). GEFs induce activation by exchanging GDP for GTP, whereas GAPs enhance the intrinsic rate of hydrolysis of bound GTP to GDP, resulting in inactivation. In cells, Rho-like GTPases exist predominantly in an inactive GDP-bound form in a complex with RhoGDI. It is assumed that RhoGDI maintains Rho-like GTPases in the cytoplasm and must dissociate to allow them to translocate to the membrane and interact with membrane-associated activators.

In previous studies, we have identified the invasion-inducing *Tiam1* gene, which encodes an activator (GEF) of the Rho-like GTPase Rac (1,2). The Tiam1 protein harbors a Dbl Homology (DH) domain flanked by a Pleckstrin Homology (PH) domain. This DH-PH combination is found in virtually all activators (GEFs), which modulate the activity of Rho-like GTPases. Tiam1 contains also a second PH domain (PHn), located N-terminally of the DH-PH unit. This PHn domain is crucial for proper membrane localization of the protein. A Coiled coil region located downstream of the PHn domain is also required for membrane localization of Tiam1 and has been shown to represent an interaction domain for other proteins. Furthermore, Tiam1 harbors a PDZ protein-interaction domain and a Ras-binding domain. The latter is thought to play a role in the direct signaling from activated Ras towards Rac. The *Tiam1* gene maps to chromosome 21q22 and is highly expressed in brain and testis and expressed at lower levels in most other tissues. *Tiam1* transcripts are found in a large number of tumor cell lines including lymphomas, melanomas, neuroblastomas and carcinomas.

Tiam1 activates Rac and overexpression of Tiam1 in NIH3T3 fibroblasts causes extensive membrane ruffling, similar to V12Rac1-expressing fibroblasts (2). Pull down assays, used to determine the activation (GTP-bound) state of Rho-like proteins, revealed that Tiam1 indeed acts as a specific activator of Rac and not Cdc42 or RhoA (3). This has further been confirmed by structure analysis of Tiam1 in association with Rac (4). PI3-kinase inhibitors abolish Tiam1-mediated Rac activation and invasion (3). Products of PI3-kinase bind to the PHn domain of Tiam1 and thereby could localize Tiam1 at the plasma membrane, where it can activate Rac. Tiam1 may also be localized by direct binding to activated Ras (5). Tiam1 is phosphorylated at threonine residues. Only membrane-localized Tiam1 is phosphorylated and is able to activate Rac, suggesting that threonine phosphorylation may play a role in activation and/or localization of Tiam1.

In epithelial carcinomas, invasion and metastasis is often associated with reduced E-cadherin-mediated cell-cell adhesion. Ectopic expression of Tiam1 in epithelial cells inhibits HGF-induced cell scattering by increasing E-cadherin-mediated adhesions. Increased Tiam1-Rac signaling also inhibits invasion and migration of fibroblastoid Ras-transformed MDCK cells by restoring E-cadherin-mediated adhesions and an epithelial phenotype (6). Interestingly,

Tiam1/Rac-induced cellular responses with respect to cell-cell adhesion and cell migration are dependent on integrin-mediated cell substrate interactions and the cell type studied. Migration of epithelial cells is determined by a balance between invasion-inhibitory cell-cell interactions and invasion-promoting cell-substrate interactions, both regulated by Tiam1-Rac signaling.

In vitro data suggest that Rac may play a role in the formation and progression of epithelial tumors *in vivo*. To study this directly, we have generated Tiam1-deficient mice using gene targeting (7). Although Tiam1 is expressed during embryonic development, *Tiam1*$^{-/-}$ mice develop, grow and reproduce normally. Compensation by other GEFs capable of activating Rac, such as Sos, Vav, Pix, Trio, SWAP-70, and Tiam2, may play a role in suppressing a developmental phenotype. In mouse skin, Tiam1 is present in basal and suprabasal keratinocytes of the interfollicular epidermis and in hair follicles. Therefore, skin tumors were initiated in wild-type and *Tiam1*$^{-/-}$ littermates by application of a two-stage chemical carcinogenesis protocol using DMBA and TPA, which invariably induces oncogenic activation of the *c-Ha-Ras* gene (8). *Tiam1*-deficient mice appeared to be resistant to the development of Ras-induced skin tumors. Moreover, the few tumors produced in *Tiam1*$^{-/-}$ mice grew much slower than tumors in wild-type mice. Analysis of *Tiam1* heterozygotes indicated that DMBA-induced tumor initiation as well as TPA-induced tumor promotion was dependent on the gene dose of *Tiam1*. Although the number of tumors in *Tiam1*$^{-/-}$ mice was small, a greater proportion progressed to malignancy, suggesting that Tiam1-deficiency promotes malignant conversion. The Rac activator Tiam1 is thus a critical regulator of different aspects of Ras-induced tumorigenesis (7).

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Defects in lymphoid, myeloid, and macrophage lineages revealed in mice congenitally lacking Vav-family proteins

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Members of the Vav family are multidomain proteins containing structural motifs characteristic of proteins involved in signal transduction including two carboxy terminal Src homology 3 (SH3) domains flanking a single SH2 domain and a Dbl homology (DH) domain, which catalyzes exchange of GDP to GTP for Rho family GTPases. While the exclusive expression pattern of Vav1, which is restricted to hematopoietic cells, suggested that Vav1 may be a critical signaling component involved in both adaptive and innate immune responses, identification and characterization of additional Vav isoforms/family members, Vav2 and Vav3, suggested a degree of functional compensation. Our lab has generated mice congenitally lacking all 3 Vav family members (triple Vav deficient (TKO)) that demonstrate the essential role of Vav proteins in development and activation of both T cells and B cells. A particular focus is on elucidation of redundant and non-redundant functions of individual Vavs in T and B cell development and activation.

We show that Vav3 is rapidly tyrosine-phosphorylated in response to BCR triggering in resting splenic B cells, while mice with combined deficiencies in Vav3 and Vav1 were markedly defective in their proliferative responses to IgM cross-linking and displayed attenuated immune responses to TI2 antigens. In addition, Vav-deficient pro-B cells displayed reduced proliferative potential in long-term IL7 cultures. Our biochemical analyses of signaling pathways triggered by IgM and TCR cross-linking including activation of tyrosine phosphorylation, phosphorylation of ERK, JNK, p38 and Akt, as well as induction of NFkB (IkB degradation) and kinetics of intracellular Ca^{++}-fluxes suggest a lineage specific functional hierarchy of Vav isoforms. Thus, both T and B lymphocytes lacking all 3 Vav isoforms show extremely severe developmental and functional defects.

Regulation of Vav proteins is very complex and involves tyrosine phosphorylation, binding to phosphatidylinositides and potentially, proteolytic cleavage. To date, the exact mode of regulation under physiologic conditions in lymphoid cells is still unknown. For example, much debate surrounds the phosphorylation of putative regulatory tyrosine residues in the acidic region of Vav1 as an inhibitory or an activating event. Structural studies have lead to speculation that tyrosines 142, 160 and 174 play a regulatory function as part of an N-terminal extension of Vav1 that occupies the GEF active site, with Y174 thought to be dominant in this context. It is thought that PIP3-induced conformational change between the PH domain and the DH domain allows tyrosines Y174, 160 and 142 in Vav1 to become phosphorylated, presumably by ZAP-70 or Lck, resulting in the activation of Vav.

Our aim is to study regulation of Vav in lymphocytes during development and activation *in vivo*. To this end, we pursued several experimental approaches including exon replacement as well as TKO bone marrow-derived hematopoietic stem cells (HSCs) based retroviral-mediated bone marrow transplantation (RVBMT), followed by generation of radiation chimera mice in wild type or in recombination-deficient (RAG) backgrounds. Here

we show that the introduction of a wild type Vav1 expression construct rescues the phenotypic defects of TKO lymphocytes providing evidence for the essential role of phosphorylation of tyrosine residues in the acidic region in the regulation of Vav1. We used RVBMT with either wild type, or mutated Vav1 expression constructs containing tyrosine to phenylalanine substitutions in positions 140, 162 and 174 and show that tyrosine 174 is essential for the regulation of Vav1 *in vivo*. Thus, unlike RV-Vav1.WT, the introduction of RV-Vav1.Y174F expression construct does not permit efficient positive selection, and does not "rescue" activation defects of Vav-deficient T cells, as assessed by induction of the CD69, actin polymerization, and blast transformation, and proliferation. Surprisingly, substitution of all three tyrosines in the "acidic loop" to phenylalanines (the Y3F mutation) has partially reversed the effects of Y174F mutation. While at present we do not understand the exact mechanism underlying the effects of these mutations, we speculate that phosphorylation of tyrosine 174 may have fundamentally distinct consequences than tyrosine phosphorylation in positions 142 and 160.

Although the exact *in vivo* function of Vav3 is unknown at present, evidence from several studies indicates that its role maybe distinct from Vav2, or Vav1. Gene knock-out studies established the importance of Vav1 and Vav2 in T and B lymphocytes. However, little is known about function(s) of recently identified Vav3 gene. While expression of Vav1 is restricted to hematopoietic cells, Vav2 is expressed more broadly. In contrast to the other family members, Vav3 is expressed at extremely low levels in most tissues. Suprisingly, we have found that the expression of Vav3 is regulated during the cell cycle, whereas enforced expression of Vav3 perturbed cytokinesis and led to the appearance of multinucleated cells.

Strikingly, Vav3 is highly expressed in osteoclasts (OCs), which are the polykaryons responsible for bone resorption, a process during which the cell's cytoskeleton undergoes re-organization, an event regulated by the Rho-family GTPases. This observation prompted us to analyze the development and function of OCs in mice congentially lacking Vav3. Notably, histomorphometric analysis of long bones from Vav3-/- and Vav1/3 double knock-out mice, but not Vav1-/-, shows dramatically increased trabecular bone volume (BV/TV) compared to WT mice, suggesting that Vav3 promotes OC formation and/or function. Thus, OCs from Vav3 and specifically Vav1/3 null mice have a strikingly abnormal phenotype in that they are smaller, irregularly shaped, less spread, and fail to resorb bone *in vitro*. Retroviral transfection of GFP-fusion Vav3, but not GFP-fusion Vav1, rescues the aberrant phenotype of the mutants. In addition, confocal analysis of cells lacking Vav3 and Vav1/3 reveals dramatic changes in the cytoskeletal organization, with failure to form functionally-important peripheral actin rings. As a consequence of the aberrant cytoskeleton, cell adhesion-mediated ERK activation and Src phosphorylation are completely abrogated in Vav3 and Vav1/3-deficient OCs. Thus Vav3, while not regulating OC differentiation, is key to controlling cytoskeletal reorganization, integrin signaling and thus bone resorption.

Role of Ras proteins in regulating Rap1 GEF localization and activity

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We and others have identified putative RalGDS/AF6 homology or Ras association (RA) domains in a number of Ras and Rap family GEFs that include MR-GEF, PDZ-GEFs 1 and 2, Epac2, Link2, phospholipase Ce, and GRASP1, reviewed in [1]. The RA domains of PDZ-GEF1 and Link2 bound to Rap1 while that of MR-GEF bound M-Ras [2]. Association with M-Ras reduced the ability of MR-GEF to increase total cellular Rap-GTP levels [2]. We have now demonstrated that the cAMP-regulated Epac2, but not Epac1, associates with H-Ras-GTP (but not with Ral, Rap1 or 2, TC21, R-Ras, Rheb, Rit, or Rin). This interaction is disrupted by mutation of Epac2's RA domain. Co-expression of Ras(61L) with Epac2 inhibits its ability to elevate total Rap1-GTP levels *in vivo*. Using confocal microscopy we have shown that GFP-Ras(61L) promotes translocation of Flag-tagged Epac2 from the cytosol to the plasma membrane, suggesting that relocalization of Epac2 upon Ras activation will result in the activation of a discrete peripheral pool of Rap1. This possibility is currently being investigated. The activation of Rap1 in this distinct subcellular locale may elicit different biological effects e.g. a small pool of phospho-Rap1 appears to be responsible for regulating Akt activity in thyroid cells in response to TSH [3]. We are using a Rap1/K-Ras chimera that preferentially targets to the plasma membrane to examine the role of Rap1 activation at this site. Interestingly Rap1A localizes to epithelial cell junctions and its activity is cell density dependent. Since PDZ-GEF complexes with MAGI and b-catenin in cell junctions, we are currently studying how this Rap GEF might be regulated by cell-cell contact. Any new findings will be reported.

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Session 5: CDC 25 GEFs
Chair: Eugenio Santos

Ras-GRF1 influence on Rac effector signaling specificity

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A growing body of evidence supports the idea that GEFs not only activate GTPases, but also contribute to signaling specificity emanating downstream from GTPases. The first examples of this phenomenon were from DH domain-containing GEFs that function to modulate Rac and CDC42 activation of JNK and PAK1 kinases (1,2). We have recently added support for this hypothesis by the identification of IB2/JIP2 as a binding partner for Ras-GRF1(3). IB2/JIP2 is a scaffold for components of the p38 MAP kinase signaling cascade, including MLK3, a known Rac effector. We showed that the binding of IB2 to Ras-GRF1 promotes the association of components of the p38 cascade with IB2 and results in the preferential activation of p38 over other Rac effectors by Ras-GRF1. In new studies, we have detected two additional binding partners of Ras-GRF1, each of which couples RasGRF1 to distinct additional Rac effector pathways. Experiments will be discussed to support the hypothesis that the binding of Ras-GRF1 to specific scaffold proteins influences the specificity of signaling through its DH-domain target Rac.

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The RasGRP family of Ras activators link phospholipid metabolism and Ras signaling

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RasGRP1 is a Ras activator in the CDC25 family that contains a C1 domain similar to those in PKC and a structure resembling a pair of EF hands. The structure of RasGRP1 suggests that it serves to link membrane receptor events, and diacylglycerol (DAG) and calcium messengers to downstream Ras signaling. Our analysis has shown that RasGRP does bind calcium and DAG analogues, such as the tumor-promoting phorbol esters. In rat2 cells, RasGRP1 translocates to the membrane in response to DAG signaling and this behavior depends on the C1 domain. Although the EF hands do bind calcium in vitro, the function of this module is unclear.

We have documented RasGRP1 expression in T cells and thymocytes. Regulation of Ras downstream of the T cell receptor (TCR) had previously been shown to involve DAG signaling events. Therefore, we questioned whether RasGRP1 constituted the link between DAG and Ras activation. We showed that stimulation of Jurkat T cells through the TCR resulted in translocation of RasGRP1 to the membrane. Furthermore, Ras activation is at least partially dependent on phospholipase C activity. Over-expression of RasGRP1 in Jurkat T cells renders the cells hyper-responsive to TCR and DAG analogue stimulation. Finally, a mouse mutant lacking RasGRP1 exhibited defects in positive selection, the earliest stage at which the TCR functions to promote thymocyte differentiation into mature T cells. The defective thymocytes also demonstrated defective Ras activation in vitro, confirming our hypothesis. RasGRP1 is expressed in some neurons and kidney cells so it must perform functions besides the transduction of TCR signals.

The RasGRP family has four members. Each has a similar domain structure but significant functional differences are apparent. RasGRP3 is expressed in B cells, suggesting that it may function downstream of the B cell receptor. As expected, when B cells are stimulated in vitro, RasGRP3 translocates to the particulate fraction. However, we also observed that RasGRP3 is heavily phosphorylated upon B cell activation. We are pursuing the hypothesis that DAG signaling co-activates both RasGRP3 and a DAG -responsive protein kinase, followed by regulatory phosphorylation of the former by the latter.

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Calcium-sensitive regulators mediate compartmentalized activation of Ras

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We have shown that N- and H-Ras are expressed on Golgi membranes as well as the plasma membrane (1). Using GFP-Raf-1-RBD, an in vivo fluorescent probe specific for activated Ras, we further demonstrated that ligation of protein tyrosine kinase receptors (PTKRs) at the cell surface stimulated rapid, transient activation of Ras at the plasma membrane and delayed, sustained activation of Ras on Golgi (2). Mitogen stimulated activation of Ras on Golgi but not on plasma membrane required Src family kinases, the tyrosine phosphatase Shp-2, and phospholipase Cgamma (PLCg).

Actiation of Src was associated with dephosphorylation of tyrosine 527 and was Shp-2-depedent. Activation of PLCg was associated with phosphorylation on tyrosine 725, a modification that required Src. These data suggest that downstream of PTKRs Shp-2 activates Src which in turn contributes to activation of PLCg. Activation of Ras on Golgi was independent of of vesicular transport suggesting that the signal was transduced by a soluble mediator, such as cytosolic Ca^{++} released upon PLCg activation. The Ca^{++} regulated Ras GEF RasGRP1 (3) translocated from the cytosol to endomembranes, including Golgi, but not plasma membrane upon stimulation of cells with growth factors. Expression of a dominant negative mutant of RasGrp selectively impaired Ras activation on Golgi but not plasma membrane. Conversely, a Ca++ activated Ras GAP, CAPRI (4) downregulated Ras at the plasma membrane but not on Golgi. Taken together, our results suggest that following growth factor stimulation Shp-2 and Src cooperate to activate PLCg that then generates DAG and IP3 that causes release of Ca++ that in turn stimultes translocation of RasGRP to Golgi and CAPRI to the plasma membrane such that Ras is simultaneously activated on the intracellular membrane compartment and deactivated at the cell surface.

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Structural and kinetic mechanisms of Vav autoinhibition and activation by tyrosine phosphorylation

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Small GTPases in the Rho family transmit signals from cell surface receptors to modulate various physiological responses including cytoskeletal reorganization leading to changes in cell shape and motility, mitogenesis and development[1,2]. Proteins containing the Dbl homology (DH) domain activate Rho GTPases by catalyzing the exchange of GDP for GTP. The mechanism of DH domain activation by upstream receptors is not well understood in most cases[3]. For the Dbl protein Vav, this mechanism involves tyrosine phosphorylation, which relieves an autoinhibitory interaction, resulting in increased nucleotide exchange activity *in vitro* and *in vivo*[4]. We have determined the structure of the minimal autoinhibited fragment of mVav1, containing the DH domain and a 20-residue N-terminal extension[5]. The structure reveals that the N-terminal extension forms a short alpha-helix, which lies in the GTPase binding site of the DH domain, explaining the lack of activity in the autoinhibited molecule. This extension contains the Tyr174 Src-family kinase recognition site, and phosphorylation of this residue, as well as truncation of the peptide, results in stimulation of GEF activity. The Tyr174 sidechain is buried in the interface between the inhibitory helix and body of the DH domain, with its phenolic hydroxyl group hydrogen bonded to a conserved, buried salt-bridge near the catalytic site. NMR spectroscopy data show that the N-terminal peptide is released from the DH domain and becomes unstructured upon phosphorylation. Thus, tyrosine phosphorylation relieves autoinhibition by exposing the GTPase interaction surface of the DH domain, which is obligatory for Vav activation.

The mechanism of activation indicated by these analyses raises a kinetic problem. Since the Tyr174 sidechain is buried in the autoinhibited structure, how can a kinase efficiently phosphorylate this site? This problem appears to be general among autoinhibitory mechanisms, where activator proteins must access sites buried in the inhibited state of their targets in order to mediate activation. We have proposed a general conceptual solution to this problem that we are currently testing with Vav. In addition to the Tyr174 site, Vav also contains two adjacent tyrosine motifs, centered at Y142 and Y160, that can be phosphorylated by Src-family kinases *in vitro* and *in vivo*[4]. These sites are also consensus binding sites for Src-family SH2 domains, and we have demonstrated that a triply phosphorylated N-terminal Vav peptide binds tightly to the SH2 domain of Src. NMR studies indicate that the Y142 and Y160 sites are largely disordered in solution in the autoinhibited state of the DH domain. Since these sites are accessible to kinases, they can be readily phosphorylated in autoinhibited DH proteins. These phosphotyrosine motifs could then form docking sites for the SH2 domains of Src-family kinases. This initial interaction of the kinase with these "access points" of Vav could convert the potentially slower phosphorylation of Y174 to an intramolecular process, enabling the kinetic barrier posed by masked binding/inhibitory elements to be overcome. Conformational fluctuations of the N-terminal extension in the autoinhibited Vav could be sufficiently rapid to give the bound kinase efficient access to the buried Tyr174 sidechain. Alternatively, binding of the kinase could increase fluctuation amplitude or rate, facilitating access. Interestingly, examination of the domain architecture of

other autoinhibited proteins suggests that access points may be a common feature of these systems, needed to overcome the kinetic barrier inherent in the autoinhibited design.

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Ras-GRF1 signaling is required for normal β-cell development and glucose homeostasis

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We have analyzed functional aspects of knockout mice strains for various Ras-GEF family members in order to ascertain the functional specificity or redundancy of their encoded cellular products. Whereas *Sos1* -/- animals died *in utero* due to placental malformation, homozygous disruption of other GEF loci (*Grf1, Grf2, Sos2*) gave rise to viable animals whose phenotypes could be analyzed.

Our analysis of *Grf1* -/- animals has generated experimental evidence suggesting a role of Ras-GRF1 in β-cell development in the pancreas. Diabetes is characterized by hyperglycemia due to insufficient insulin production by pancreatic β-cells. Development of diabetes generally reflects an inadequate mass of insulin producing cells. β-cell proliferation and differentiation are regulated by a variety of growth factors and hormones, including IGF-I. GRF1 is a Ras-guanine nucleotide exchange factor known previously for its restricted expression in brain and its role in learning and memory. Here we demonstrate that GRF1 is also expressed in pancreatic islets. Interestingly, our GRF1-deficient mice exhibit reduced body weight, hypoinsulinemia and glucose intolerance owing to a reduction of β-cell mass. Whereas insulin resistance is not detected in peripheral tissues, GRF1-knockout mice are leaner due to increased lipid catabolism. The reduction in circulating insulin does not reflect defective glucose sensing or insulin production but results from impaired β-cell proliferation and reduced neogenesis. IGF-I treatment of isolated islets from GRF1 knockouts fails to activate critical downstream signals such as Akt and Erk. The observed phenotype is similar to manifestations of preclinical type 2 diabetes. Thus, our observations demonstrate a novel and specific role for Ras-GRF1 pathways in the development and maintenance of normal β-cell mass and function.

POSTERS

Activation of Ras in the Golgi is mediated by CalDAG proteins

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CalDAGs, also known as RasGRPs, are a novel family of proteins that work as exchange factors for Ras. All CalDAG proteins described to date (CalDAGI, II, III and RasGRP4) share the same structural domains: a catalytic domain for the exchange activity of Ras and/or Rap, a calcium binding domain, and a DAG binding domain. Recent studies have revealed that RasGRP is a key molecule in thymocyte differentiation and T-cell activation, linking the T-cell receptor and PLC-γ to Ras signalling. Optimal Ras activation in lymphoid cells also depends on Vav proteins since the absence of Vav abolishes the activation of Ras. Our study of the mechanism for activation of Ras by Vav has revealed a novel cross-talk mechanism between the Ras and the Rac pathways based on the direct stimulation of CalDAGs by Vav. This mechanism involves the active translocation of CalDAG to peripheral structures enriched in F-actin and the activity of PLC-γ.

We have observed a marked different subcellular localization between CalDAG isoforms in COS cells. CalDAGI localizes in the cytosol whereas CalDAGII localizes to the ER and Golgi. This subcellular localization depends on the integrity of the DAG binding domain and is regulated by PMA and EGF. It has been recently described that Ras can be activated in the Golgi. The ER and Golgi localization of CalDAGII suggested a role of this exchange factor in the activation of Ras specifically in these subcellular compartments. Using the Raf-1-RBD-GFP as a fluorescent probe to detect Ras activation, we observed that CalDAGII induces an increase in activated Ras mainly in the Golgi. By pull-down and focus formation assays using mutated forms of Ras that target this GTPase to endomembrane compartments (ER and Golgi) we also observed the activation of these Ras mutants by CalDAGII.

Our data support the hypothesis of a specific role of the different isoforms of CalDAG proteins on the activation of Ras at specific subcellular compartments, and corroborates the complexity of Ras signalling in the cell.

Role of DGKα in cytoskeletal reorganization

Silvia Carrasco and Isabel Mérida

Diacylglycerol Kinase (DGK) phosphorylates diacylglycerol (DAG) to phosphatidic acid (PA), two lipids with recognised biological activity. Recent work from our laboratory has demonstrated that, in lymphocytes, membrane localization and activation of DGKα acts as a "switch off" signal for Ras activation, mediated by localization to the plasma membrane of Ras-GRP. Activation of DGKα not only attenuates DAG levels but also induces an elevation on the membrane levels of PA. This lipid messenger has been suggested to participate in actin polymerization and stress fiber formation in several cell types.

We have generated a membrane bound form of DGKα by fusing the cDNA of DGKα to a myristoilated GFP (MyrGFP-DGKα). We have then used this construct, that induces an increase in membrane PA levels, to investigate changes in lymphocyte cell morphology. Similar experiments have been carried out in endothelial cells, where DGKα-dependent PA generation has been postulated to participate in cytoskeletal reorganization. In exponentially growing endothelial cells, the expression of MyrGFP-DGKα induces changes in cell morphology that suggest lack of cell adhesion. However the transfection of this construct apparently does not reduce the total number of attached cells. We have next investigated the effect of constitutive localization to the membrane of DGKα in PDGF-stimulated cells. Our results suggest that membrane localization of DGKα affects the correct regulation of cytoskeletal reorganization. The possible mechanisms underlying this effect will be discussed.

Activation of JNK by EPAC is independent of its activity as a Rap Guanine Nucleotide Exchanger

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Guanine Nucleotide Exchange Factors (GEFs) and their associated GTP binding proteins (G-proteins) are key regulatory elements in the signal transduction machinery that relays information from the extracellular environment into specific intracellular responses. The MAPK cascades represent ubiquitous downstream effector pathways. We have previously described that, analogous to the Ras-dependent activation of the Erk-2 pathway, members of the Rho family of small G-proteins activate the JNK cascade when GTP is loaded by their corresponding GEFs. Searching for novel regulators of JNK activity we have identified EPAC as a strong inducer of JNK-1. EPAC (Exchange Protein Activated by cAMP) is a member of the growing family of GEFs that specifically display exchange activity on Rap, which are members of the Ras family of small G-proteins. We report here that while EPAC activates the JNK several fold, a constitutively active (G12V) mutant of Rap1b does not, suggesting that EPAC Rap-GTP is not sufficient to transduce EPAC-dependent JNK activation. Moreover, EPAC signaling to the JNKs was not blocked by inactivation of endogenous Rap suggesting that Rap activation is not necessary for this response. Finally, domain deletions mutant analysis shows that the catalytic GEF domain is dispensable for EPAC-mediated activation of JNK while a region overlapping the REM domain is critical. We conclude that EPAC signals to the JNK cascade through a previously undescribed mechanism that does not involve GDP/GTP interchange upon its small G-protein counterpart, which represents a novel way to activate the JNK.

The neuron specific Ras-exchange factor RasGRF1 assembles with polymerized tubulin: microscopy analysis and biochemical studies

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CDC25Mm/RasGRF1 is a Ras guanine nucleotide exchange factor, expressed only in neurons of the central nervous system (1). It has a modular structure with a catalytic domain in the C-terminal region, a Pleckstrin homology domain a coiled coil and a calmodulin binding IQ domain in the N-terminal region. In addition a DH-PH module present in the central part of the molecule is responsible for the guanine nucleotide exchange activity on Rac (2).

In mouse brain part of RasGRF1 is localized at the synaptic junctions, being particularly enriched in the postsynaptic densities (3). In addition it is also present in the high-speed brain supernatant.

To further investigate the *in vivo* localization of RasGRF1 we have prepared constructs coding for fusion proteins containing the first 239 N-terminal amino acids (that is the PH1, the coiled coil and the IQ domain) fused with either their C-or N- terminal end to GFP and expressed them in NIH 3T3 fibroblasts and HEK 293 cells. Fluorescence microscopy analyses indicate that both constructs partially localize at the plasma membrane. Moreover the constructs with the free N-terminal and the full length Ras-GRF1 molecule colocalize also with microtubules.

Association of RasGRF1 with polymerized tubulin is also detected using an *in vitro* assay that allows microtubules assembly in extracts of adult mouse brain. Further studies are in progress to investigate the molecular bases of this association.

These results are relevant particularly in view of recent findings showing interaction of RasGRF1 with JIP2 (4) a scaffold protein that connects signaling molecules to kinesin motor proteins.

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Getting to know Ral new approach

Hernández, M. and Bos J.L.

Ral, is a small GTPase of the Ras family implicated in the control of cell proliferation, differentiation, cytoskeletal organization and vesicular transport. Like other members of this family, Ral is regulated by guanine-nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs catalyze the dissociation of the GDP bound to the GTPase, allowing it to bind to the GTP, resulting in the GTPase active form.

So far, various Ral-specific GEFs have been identified: RalGDS, Rgl, Rlf and RalGEF2. The first three can directly bind to GTP-bound Ras for its activation. Regulation of RalGEFs is less well understood, but this factor may account for calcium-dependent activation of Ral.

Since both Ras and increases in intracellular calcium activate multiple signal transduction pathways, we are developing tool to specifically induce Ral activity. In one of the approaches taken, we have linked the cAMP-dependent regulatory domain of the Rap1-specific GEF, EPAC, to the catalytic domain of RalGDS. Such a chimearic protein is expected to be sensitive to stimulation with cAMP-analogues. To further increase specificity, we are making use of cAMP-analogues, which are specific for EPAC and do not affect the other major target for cAMP, namely, PKA.

Progress in this approach will be presented in a poster in the workshop.

Phosphoinositide 3-Kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1

Metello Innocenti, Emanuela Frittoli, Isabella Ponzanelli, Pier Paolo Di Fiore, and Giorgio Scita

Class I Phosphoinositide 3-kinases (PI3Ks) are implicated in many cellular responses controlled by receptor tyrosine kinases (RTKs), including actin cytoskeletal remodeling. Within this pathway, Rac is a key downstream target/effector of PI3K. How the signal is routed from PI3K to Rac is however unclear. One possible candidate for this function is the Rac-activating complex Eps8-Abi1-Sos1, which possesses Rac-specific GEF (Guanine nucleotide Exchange Factor) activity. Here we show that Abi1 (also known as E3b1) recruits PI3K, via p85, into a multimolecular signaling complex, which includes Eps8 and Sos-1. The recruitment of p85 to the Eps8-Abi1-Sos-1 complex and phosphatidylinositol 3, 4, 5 phosphate (PIP3), the catalytic product of PI3K, concur to unmask its Rac-GEF activity *in vitro*. Moreover, they are indispensable for the activation of Rac, and Rac-dependent actin remodeling *in vivo*. Consistently, upon growth factor stimulation endogenous p85 and Abi1 colocalize into membrane ruffles and cells lacking p85 fail to support Abi1-dependent Rac activation. Our results define a mechanism whereby propagation of signals, originating from RTKs or Ras and leading to actin reorganization, is controlled by direct physical interaction between PI3K and a Rac-specific GEF complex.

Functional analysis of the UNC-73B PH domain demonstrates its role in RAC activation

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The *unc-73* gene from *C.elegans* has been shown to play an important role in axon guidance and cell motility in the developing nervous system. One product from this gene is UNC-73B, a protein that contains a number of protein modules, including a Dbl homology (DH) domain, which is a specific activator of the Rac GTPase and a Pleckstrin homology domain, a signaling module often involved in intracellular membrane targeting. The typical pairing of DH/PH domains may play a specific role in regulating GEF activity.

In order to gain an understanding of the involvement of the PH domain on the activity of UNC-73B, we carried out biochemical and transgenic experiments in *C.elegans* with constructs containing mutations within the PH domain. Using a dot blot technique, we demonstrate that the WT PH domain interacts with very little specificity towards any phosphorylated phosphoinositide. A double mutant, Lys1420/R1422 to Glu/Glu, was unable to bind any phospholipid tested. However, this mutation had no effect on the *in vitro* catalytic activity of the DH domain. Another mutant, Trp1502 to Ala, which should cause a destabilization of the PH domain, was able to interact with phosphorylated phosphoinositides. In this case, the GEF activity of the protein was abolished. Minigenes containing these mutations were microinjected into *C.elegans*, and transferred to *unc-73(e936)* mutant worms. Both set of mutations failed to rescue the axon guidance phenotype present in these worms. These results indicate that the PH domain plays multiple roles in stabilizing the DH domain structure, in interacting with Rac along with the DH domain and in localizing UNC-73B to the plasma membrane.

Calmodulin prevents activation of Ras by PKC in 3T3 fibroblasts

Cristina López Alcalá, Priam Villalonga, Joan Gil*, Oriol Bachs and Neus Agell

We have previously shown that calmodulin negatively regulates Ras activation in fibroblasts. Hence, anti-calmodulin drugs (such as W13, trifluoroperazine or W7) are able to induce Ras/ERK pathway activation under low levels of growth factors. We show now that cell treatment with PKC inhibitors abolishes W13-induced activation of Ras, Raf-1 and ERK. In consequence, PKC activity is essential to achieve the synergism between calmodulin inhibition and growth factors to activate Ras. Furthermore, whereas activation of PKC by TPA does not induce Ras activation in 3T3 cells, activation is observed if calmodulin is simultaneously inhibited. This indicates that calmodulin is preventing Ras activation by PKC.

Treatment of cells with EGF- or PDGF-receptor tyrosine kinase inhibitors does not abrogate activation of Ras by calmodulin inhibition. This implies that EGF- and PDGF-receptor tyrosine kinase activities are dispensable to activate Ras by TPA plus W13 and, therefore, Ras activation is not a consequence of the transactivation of those receptors by the combination of the anti-calmodulin drug plus TPA. Furthermore, K-Ras, the isoform previously shown to bind to calmodulin, is the only one activated by TPA when calmodulin is inhibited. These data suggest that the direct interaction between K-Ras and calmodulin may account for the inability of PKC to activate Ras in 3T3 fibroblasts. *In vitro* experiments showed that phosphorylation of K-Ras by PKC was inhibited by calmodulin, suggesting that calmodulin-dependent modulation of K-Ras phosphorylation by PKC could be the mechanism underlaying K-Ras activation in fibroblasts treated with TPA plus W13.

Specificity for the inhibitory effects of H Ras, K Ras and N Ras N17 dominant inhibitory mutants is related to their membrane microlocalization

Matallanas D., Arozarena I., Aaronson D.S., Berciano M.T., Pellicer A., Lafarga M., and Crespo P.

Ras GTPases are essential mediators in signaling pathways that convey extracellular signals from surface to the interior of the cell. The three ras genes have great simiralities, regardless their functions may not be completly redundant. Multiple strategies have been utilized to unveil the functions of Ras proteins in cells. In this work, we have used N17 dominant inhibitory mutants to do a comparative study of their inhibitory functions. We demonstrate that H, K, and N-Ras N17 mutants exhibits markedly distinct specifities on their inhibitory functions. H-Ras N17 can inhibit the activation of all Three isoforms. K- Ras N17 specifically inhibits the activation of K-Ras. Likewise, N-Ras N17 can only inhibit N-Ras activation. These results can be axplained in light of the recent data on the compartmentalization of Ras proteins. In This respect we present for the first time a detailed description of N-Ras cellular microlocalization.

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The LPA receptor Edg-2/LPA1 regulates cell spreading and migration : a role for Tiam1/Rac1 signalling

Cristina Olivo, Frank N. van Leeuwen, *, Shula Grivell*, and Wouter H. Moolenaar* John G. Collard

Lysophosphatidic acid (LPA) is a bioactive lysophospholipid that acts via G protein-coupled receptors (GPCRs). Three distinct G protein-coupled receptors for LPA have been identified, LPA1, LPA2 and LPA3 (Edg2, Edg4 and Edg7, respectively), with LPA1/Edg2 being the of these receptor family. (Contos et al., 2000). LPA , via GPCR, induces diverse cellular responses, from rapid morphological changes to cell proliferation and survival (Moolenaar, 1999). LPA, for instance, activates the mitogenic Ras-ERK1/2 cascade via Gi (Kranenburg, 2001) and induces cell rounding and neurite retraction via G12/13-mediated activation of RhoA, (Kranenburg et al., 1999). However, it is still unclear the role of LPA on cell motility and migration in the transition of tumors from a non-invasive to an invasive and metastatic phenotype. LPA stimulates the invasion of tumor cells (Stam et al., 1998) and promotes wound healing both *in vitro* and *in vivo* (Sturm, 1999; Lee et al., 2000). In general, cell migration is driven by Rho family GTPases, notably RhoA, Rac1 and Cdc42: Rac1 regulates lamellipodia protrusion and forward movement, RhoA mediates contraction of the rear end of migrating cells, and Cdc42 establishes cell polarity.

Here we analyze the LPA1 receptor-signaling pathways, particularly those that involve Ras and Rho family GTPases. We show that, in addition to transiently activating RhoA, the LPA1 receptor mediates activation of Rac to induce lamellipodia formation, cell spreading and migration, and we establish a pathway that couples the LPA1 receptor the invasion-inducing Rac activator Tiam1.

The Rho exchange factor Net1 is regulated by nuclear sequestration

Anja Schmidt and Alan Hall

The Net1 gene, encoding for a guanine nucleotide exchange factor (GEF) specific for the small GTPase Rho, was originally isolated as an oncogene from neuroepithelioma DNA using a focus formation assay in NIH3T3 fibroblasts. Oncogenic activation of Net1 occurs by truncation of the N-terminal part of the protein, which functions as a negative regulatory domain. Here, we have investigated the mechanism of Net1 regulation via its N-terminus. We find that Net1 localizes to the nucleus whereas oncogenic Net1 is found in the cytoplasm. Nuclear import of Net1 is mediated by two nuclear localization signals (NLSs) present in the N-terminus, and deletion or mutation of these leads to an increase in cytoplasmic localization and results in activation of Rho. In addition, we show that the pleckstrin homology (PH) domain of Net1 has at least two activities. First, it acts as a nuclear export signal, and secondly, it is required for GEF activity of Net1 once in the cytoplasm. Together our results suggest that Net1 can shuttle in and out of the nucleus, and that activation of Rho by Net1 is controlled by changes in its subcellular localization.

Ras activity towards p21WAF1 occurs through MAPK pathway and is inhibited by cMyc

Vaqué J.P., Delgado M.D., Mauleón I., Ajenjo N. and León J.

Chronic myeloid leukemia (CML) is a neoplasia where no Ras activating mutation has been reported. We recently demonstrated that overexpression or a mutated version of H, N, K-Ras, inhibit K562 (CML derived) cells, by a mechanism dependent on p21 WAF1 and independent on p53, p15 INK4b, p16 INK4a and p19 ARF. Oncogene (2000) 19, 783-790. Using the inhibitor PD98059, and effector-specific Ras mutants, we have found that this Ras-mediated p21WAF1 activation and the subsecuent growth inhibition occurs through a MAPK-dependent pathway.

We and others have previously demonstrated that cMyc inhibits p21 WAF1 activation through p53 and other stimuli. Here we demonstrate that cMyc inhibits H, N, and K-Ras-mediated p21 WAF1 activation, without affecting MAPK activity. Moreover this Ras inhibition by cMyc depends on its transcriptional activity, shown by using cMyc deletion mutants lacking the transactivation domain and by coexpression of cMyc with Max or Mad proteins, which regulate cMyc transcriptional activity. Interestingly only Mad1 was able to revert cMyc inhibition over Ras activity.

These results show that cMyc, can inhibit Ras signaling, downstream of MAPK, at the level of genetic regulation in a Ras-related non-proliferative enviroment.

RhoG regulates gene expression and the actin cytoskeleton in lymphocytes

Elena Vigorito, Gina Doody, Simon McAdam, Phillipe Fort and Martin Turner

RhoG, a member of the Rho family of GTPases, has been implicated as a regulator of the actin cytoskeleton. In this study we show a novel function for the small GTPase RhoG on the regulation of the Interferon-g promoter and Nuclear Factor of Activated T cells (NFAT) gene transcription in lymphocytes. Optimal function of RhoG for the expression of these genes requires a calcium signal, normally provided by the antigen receptor. In addition, RhoG potentiation of NFAT requires the indirect activity of Rac and Cdc42; however, pathways distinct from those activated by Rac and Cdc42 mediate RhoG activation of NFAT-dependent transcription. Using effector domain mutants of RhoG we found that its ability to potentiate NFAT-dependent transcription correlates with its capacity to increase actin polymerisation, supporting the suggestion that NFAT-dependent transcription is an actin dependent process.

RhoG also promotes T cell spreading on fibronectin, a property that is independent of its ability to enhance NFAT-dependent transcription. Hence, these results implicate RhoG in leukocyte trafficking and the control of gene expression induced in response to antigen encounter.

P-Rex1, a novel PtdIns(3,4,5)P3- and Gbg-regulated guanine-nucleotide exchange factor for Rac

Heidi C. E. Welch, Christian D. Ellson, John Coadwell, Hediye Erdjument-Bromage, Paul Tempst, Phillip T. Hawkins, Len R. Stephens

Rac proteins, a subfamily of the Rho-family of small GTPases, are key regulators of many cellular responses. These range from universally important responses such as transcriptional activation, definition of cell shape (lamellipodia formation, membrane ruffling) and cell motility, to neutrophil-specific responses such as the formation of reactive oxygen species (ROS) by the NADPH oxidase. Rac activation has been shown to be under the control of phosphoinositide 3-kinase (PI3K) in many cases, and the Inositide Laboratory has been interested in the mechanisms of activation of Rac by PI3K for several years. We have shown that PI3K activates a Rac-guanine nucleotide exchange factor (GEF) activity independently of PKB in PDGF receptor-dependent signalling and that Rac is partially regulated by PI3Kg in the pathway leading to ROS formation in neutrophils. The enzyme(s) that conveys the activation was unknown but the simplest explanation of these data was that a Rac-GEF which is substantially activated by the lipid product of PI3K activity, PtdIns(3,4,5)P3, is responsible. We established an assay for such activity and found a major PtdIns(3,4,5)P3-sensitive Rac-GEF activity in pig neutrophil cytosol. This activity was purified by conventional column chromatography. The enzyme is a highly abundant, novel 185 kD protein that we named P-Rex1 (for PtdIns(3,4,5,)P3-dependent Rac exchanger) and is the major PtdIns(3,4,5)P3-sensitive activator of Rac in neutrophil cytosol. We have cloned human P-Rex1 from leukocyte and spleen cDNA libraries. It contains a typical Rho-GEF domain and tandem PH domain, two DEP and two PDZ domains and significant similarity over its C-terminal half to Inositol Polyphosphate 4-Phosphatase. Northern blots showed that P-Rex1 is expressed mainly in peripheral blood leukocytes and brain, less in spleen and lymph nodes and much weaker in most other tissues. The Rac-GEF activity of recombinant P-Rex1 is directly and substantially activated by PtdIns(3,4,5)P3 both *in vitro* and *in vivo*. Earlier results that had implied the presence of an unknown Gß?-regulated Rac-GEF activity in neutrophil cytosol and the fact that P-Rex1 has two DEP domains led us to study the potential involvement of heterotrimeric G proteins in P-Rex1 regulation. We found that P-Rex1 is also directly and strongly activated by Gß? subunits, and this activation can occur synergistically with the activation by PtdIns(3,4,5)P3. Treatment of neutrophil-differentiated myeloid HL60 or NB4 cells with P-Rex1 antisense oligonucleotides inhibits partially G-protein-coupled ROS formation by the NADPH oxidase. Together, our results define P-Rex1 as a major link between G proteins, PI3K and Rac in neutrophils.

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Organizers: P. D. Coleman, F. Mora and M. Nieto-Sampedro.

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Organizers: J. A. Cidlowski, R. H. Horvitz, A. López-Rivas and C. Martínez-A.

*30 **Workshop on Resistance to Viral Infection.**
Organizers: L. Enjuanes and M. M. C. Lai.

31 **Workshop on Roles of Growth and Cell Survival Factors in Vertebrate Development.**
Organizers: M. C. Raff and F. de Pablo.

32 **Workshop on Chromatin Structure and Gene Expression.**
Organizers: F. Azorín, M. Beato and A. P. Wolffe.

*33 **Workshop on Molecular Mechanisms of Synaptic Function.**
Organizers: J. Lerma and P. H. Seeburg.

*34 **Workshop on Computational Approaches in the Analysis and Engineering of Proteins.**
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35 **Workshop on Signal Transduction Pathways Essential for Yeast Morphogenesis and Cell Integrity.**
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Organizers: E. Coen, Zs. Schwarz-Sommer and J. P. Beltrán.

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38 **Workshop on Immunodeficiencies of Genetic Origin.**
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39 **Workshop on Molecular Basis for Biodegradation of Pollutants.**
Organizers: K. N. Timmis and J. L. Ramos.

*40 **Workshop on Nuclear Oncogenes and Transcription Factors in Hematopoietic Cells.**
Organizers: J. León and R. Eisenman.

*41 **Workshop on Three-Dimensional Structure of Biological Macromolecules.**
Organizers: T. L Blundell, M. Martínez-Ripoll, M. Rico and J. M. Mato.

42 **Workshop on Structure, Function and Controls in Microbial Division.**
Organizers: M. Vicente, L. Rothfield and J. A. Ayala.

*43 **Workshop on Molecular Biology and Pathophysiology of Nitric Oxide.**
Organizers: S. Lamas and T. Michel.

*44 **Workshop on Selective Gene Activation by Cell Type Specific Transcription Factors.**
Organizers: M. Karin, R. Di Lauro, P. Santisteban and J. L. Castrillo.

45 **Workshop on NK Cell Receptors and Recognition of the Major Histocompatibility Complex Antigens.**
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48 **Workshop on G-Proteins: Structural Features and Their Involvement in the Regulation of Cell Growth.**
Organizers: B. F. C. Clark and J. C. Lacal.

*49 **Workshop on Transcriptional Regulation at a Distance.**
Organizers: W. Schaffner, V. de Lorenzo and J. Pérez-Martín.

50 **Workshop on From Transcript to Protein: mRNA Processing, Transport and Translation.**
Organizers: I. W. Mattaj, J. Ortín and J. Valcárcel.

51 **Workshop on Mechanisms of Expression and Function of MHC Class II Molecules.**
Organizers: B. Mach and A. Celada.

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143 **Workshop on Engineering RNA Virus Genomes as Biosafe Vectors.**
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