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Ras and Rho GTPases: A Family Reunion

Review

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Introduction

GTPases of the Ras superfamily act as molecular switches to control a wide range of essential biochemical pathways in all eukaryotic cells. Among the 60 or so that have been identified so far in mammalian cells, the Ras and the Rho families are of special interest since they couple intracellular signal transduction pathways to changes in the external environment. Like all GTPases, they exist in an inactive (GDP-bound) and an active (GTP-bound) conformation. Guanine nucleotide exchange factors (GEFs) catalyze the release of GDP, allowing GTP to bind (its intracellular concentration is higher than that of GDP). In their active, GTP-bound state, Ras and Rho GTPases interact with target proteins to promote a cellular response. Finally, an intrinsic GTPase activity, catalyzed further by GTPase activating proteins (GAPs), completes the cycle and the GTPase returns to its inactive, GDP-bound state. Figure 1 shows some of the upstream GEFs, downstream targets, and GAPs for two examples, Ras itself, and Rac, a member of the Rho family.

At least three striking features have emerged from the analysis of Ras and Rho GTPases: (1) the diversity of membrane receptors and upstream regulators that can activate these GTPases, (2) the diversity of cellular targets that can interact with an individual GTPase, and (3) the extensive cross-talk and cooperation that exists between GTPase-regulated signal transduction pathways. The first two features have recently been reviewed (Van Aelst and D'Souza-Schorey, 1997; Campbell et al., 1998: Bishop and Hall, 2000). Here, we will focus specifically on the growing number of examples, obtained at both the molecular and cellular level, of the coordinated activation and the functional cooperation between members of the Ras and Rho GTPase families in animal cells.

The physiological significance of cross-talk between Ras and Rho family GTPases has been most clearly established through genetic analysis of budding and fission yeast. In *S. cerevisiae* Bud1p (also known as Rsr1p), a small Ras-like GTPase related to mammalian Rap1, is required to define the position of the new bud. Cdc42p, a Rho-like GTPase, is required to promote the assembly of bud components at that site. It is perhaps not surprising, therefore, that there should be a close temporal and spatial link between these two small GTPases, and indeed, the active form of Bud1p interacts directly with Cdc24p, a GEF for Cdc42p (Park et al., 1997). In vitro, this interaction is not sufficient to stimulate the GEF activity of Cdc24p, and one functional outcome of this interaction in vivo, therefore, is likely to be the localization of Cdc24p to the presumptive bud site (Zheng et al., 1995). In *S. pombe*, normal cell morphology is dependent on both ras1 and cdc42 functions and genetic and biochemical analysis has revealed that ras1 interacts directly with scd1, the GEF for cdc42 (Chang et al., 1994). In animal cells, it has been recognized for quite some time that Ras and Rho GTPases regulate an overlapping set of cellular responses, the best characterized of which are gene expression, cellular proliferation, and actin-based cell motility. However, the elucidation of the molecular basis for the cross-talk between Ras and Rho GTPases has awaited the delineation of the signaling events controlled by each family. With this knowledge in hand, it is now feasible to begin to define the biochemical and physiological links between these two families.

Molecular Mechanisms of Cross-Talk

Although diverse in nature, the molecular events identified thus far as mediators of cross-talk between Ras and Rho GTPases can be broadly classified into two mechanistic frameworks: the branching of upstream signals, referred to here as signal divergence, and coordinated regulation of downstream functions, referred to here as signal convergence (Figure 2). The central role played by GEFs and effectors in these regulatory processes is a clear theme to emerge from the analyses of functional interactions between Ras and Rho GTPases.

Signal Divergence

The activation of Ras and Rho GTPases in animal cells by extracellular stimuli is mediated by GEFs. The Cdc25 domain originally identified in yeast and subsequently found in at least a dozen mammalian proteins defines a family of GEFs active on members of the Ras family, while the DH/PH motif, originally identified in the mammalian Dbl onco-protein and in yeast cdc24p defines a family of GEFs (currently over 35 in mammals) active on the Rho family. The number of GEFs clearly exceeds the number of GTPases, and while the reasons remain speculative, it is likely to reflect, in part, the ability of a single stimulus to activate different subsets of GTPases depending on the cell type and the biological context. There are numerous examples of this type of signal divergence.

The addition of insulin to cultured fibroblasts leads to the rapid activation of Ras and Rac and inhibition of either GTPase does not interfere with activation of the other (Ridley et al., 1992). In lymphocytes, stimulation of the T cell receptor (TCR) appears to activate Ras and

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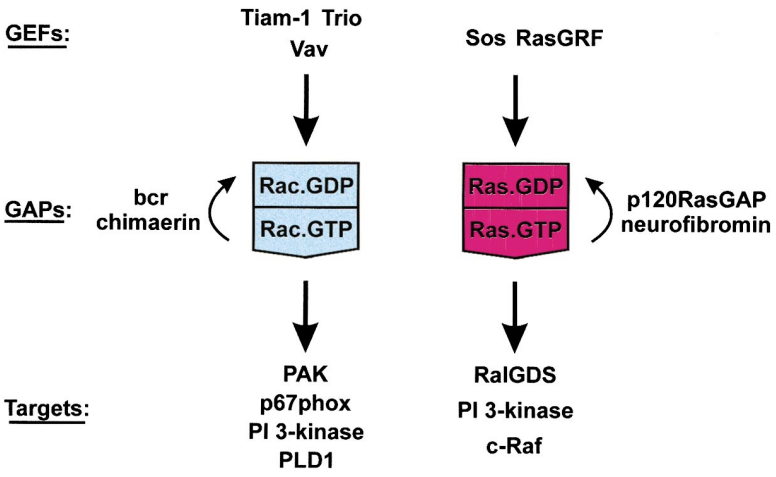

Figure 1. Ras and Rac Interact with a Large Number of Cellular Proteins

Ras and Rac (examples of the Ras and Rho families, respectively) interact with multiple GEFs, GAPs, and target (effector) proteins. The figure shows only a few examples of the many interacting proteins so far identified.

Rac independently, as does cross-linking of the FcεR1 receptor in mast cells (Holsinger et al., 1998; Turner et al., 1998). Such parallel activation of small GTPases could most obviously occur through receptor recruitment of multiple exchange factors (Figure 2), and further analysis in lymphocytes has revealed that Sos (a Ras exchange factor) and Vav (a Rac exchange factor) are both recruited to the TCR by an associated nonreceptor tyrosine kinase, ZAP-70 (Salojin et al., 2000). Ras and Rac are also activated after engagement of integrins by extracellular matrix molecules (Schlaepfer et al., 1994, Clark et al., 1998; Price et al., 1998). Ligand binding to integrins leads to the assembly of multimolecular complexes containing Sos and Vav. The recruitment of Sos to these complexes can be mediated by focal adhesion kinase (FAK) or the adaptor protein Shc, while the recruitment of Vav may depend on the nonreceptor tyrosine kinase Syk or on the assembly of the adaptor complex p130Cas/CrkII/DOCK180 (Schlaepfer et al., 1994; Wary et al., 1996; Lin et al., 1997a; Kiyokawa et al., 1998; Miranti et al., 1998; Renshaw et al., 1999).

Heptahelical receptors can similarly lead to the parallel activation of Ras and Rho GTPases, and interestingly, this is mediated by another superfamily of GTPases, the heterotrimeric G proteins. The best understood pathway linking this class of receptor to a small GTPase is through G_{13}. The molecular mechanism was nicely elucidated when it was shown that the α subunit of G_{13} interacts directly with an RGS-like sequence in p115RhoGEF and thereby stimulates nucleotide exchange on Rho (Hart et al., 1998). Several corresponding components of this pathway have been identified genetically in the gastrulation pathway of *Drosophila,* adding weight to the physiological importance of this link between a G protein and a small GTPase (Haecker and Perrimon, 1998). LPA (lysophosphatidic acid) activates Rho through this G_{13}/p115RhoGEF pathway, but in addition, it activates Ras and the ERK MAP kinase pathway. This effect appears to be mediated independently by a different G protein, G_i, since unlike Rho, Ras activation is pertussis toxin sensitive (Luttrell et al., 1996). Further analysis has revealed that it is the dissociated βγ subunits of G_i that lead to increased GTP levels on Ras by stimulating c-Src and recruiting Sos to the plasma membrane via an adaptor protein, Shc (Crespo et al., 1994; Luttrell et al., 1996).

Ras and Rho GTPases can be activated in series, such that one small GTPase stimulates GTP loading on another. This appears to be a common mechanism of signal divergence within the Rho and Ras subfamilies. Cdc42 is a strong activator of Rac in many cell types, while Rac has been reported to activate or inhibit Rho to varying degrees (Ridley et al., 1992; Nobes and Hall, 1995; Sander et al., 1999). The biochemical events mediating cross-talk between members of the Rho family is not known. Similarly, Ras activates Ral, but in this case, a family of Ral exchange factors has been identified that are regulated by direct interactions with GTP-bound Ras (Wolthuis and Bos, 1999). However, cross-talk can also occur between the two families, and constitutively activated Ras, for example, is a potent activator of Rac (Ridley et al., 1992). No clear example has yet been reported of a Rho GTPase activating a Ras GTPase.

A further indication that Ras and Rho GTPases can

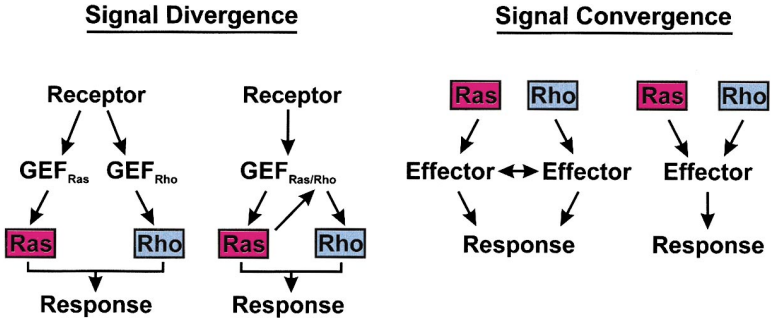

Figure 2. Mechanisms of Cross-Talk between Ras and Rho GTPases

The activities of Ras and Rho GTPases can be coordinated by two classes of regulatory mechanisms, one involving the utilization of GEFs (Signal Divergence) and the other the utilization of effectors (Signal Convergence). In this diagram, Ras and Rho denote members of the Ras and Rho families, respectively, and $GEF_{Ras/Rho}$ denotes bifunctional guanine nucleotide exchange factors. Specific examples for each mechanism are provided in the text.

be coordinately regulated is that two important Ras GEFs, Sos and Ras-GRF, contain a Rho GEF (DH/PH) domain. Sos stimulates nucleotide exchange on Ras downstream of a wide variety of plasma membrane receptors in mammalian cells and in response to tyrosine kinase receptors during embryonic development in *Drosophila.* The Cdc25-related GEF domain of Sos is coupled to membrane receptors through C-terminal proline-rich motifs that interact with adaptor proteins such as Grb2 and Shc. The physiological role of the N-terminal DH/PH motif of Sos has not been clearly established, but ectopic expression of the DH domain in cells leads to activation of Rac (Nimnual et al., 1998). Interestingly, the tandem DH/PH motif expressed in cells is inactive unless coexpressed with Ras, and this has led to the suggestion that Sos stimulates Ras and then Ras, perhaps through PI 3-kinase, activates the Sos DH/PH domain leading to Rac activation (Nimnual et al., 1998). The GEF activity of the DH domain of Vav, an activator of Rho GTPases, has been shown to be regulated by the binding of PI 3-kinase products to the neighboring PH domain, providing another potential pathway for the sequential activation of Ras and Rho GTPases (Han et al., 1998). Recently, two other adaptor-like proteins, Eps8 and E3b1, have been implicated in coupling Ras to Rac via Sos (Scita et al., 1999). In fibroblasts derived from Eps8 knockout mice, Rac cannot be activated by constitutively activated Ras. Furthermore, E3b1, a protein that binds to the SH3 domain of Eps8, interacts directly with Sos, and when these three proteins (Eps8, E3b1, and Sos) are expressed together, they can be coprecipitated from cells. The expression of the Ras exchanger Ras-GRF2, which, like Sos, contains both a Cdc25 and a DH/PH domain, induces the activation of both Ras-dependent and Rac-dependent pathways (Fan et al., 1998). The Ras-GRF2-mediated activation of Rac is dependent on its DH domain but is not sensitive to the PI-3 kinase inhibitor wortmannin, suggesting that the activation of Rac by Ras-GRF2 and Sos might be achieved through different mechanisms.

Signal Convergence

The ser/thr kinase PAK has emerged as a molecule that can link Rac and Ras signaling by converging on the ERK MAP kinase pathway. Receptor-mediated activation of Raf, the upstream MAP kinase kinase kinase in the ERK cascade, is Ras-dependent and involves recruitment of the kinase to the plasma membrane. Several groups have reported synergism between Rho GTPases and this pathway. It is widely reported that overexpression of a Rho GTPase cannot in itself lead to ERK activation, however, dominant-negative Rac can block Ras-dependent ERK activation in 293 cells (Frost et al., 1996). Furthermore, Rac or Cdc42 can synergize with Raf to promote ERK activation and this synergy has been reported to occur at the level of the MAP kinase kinase, MEK1 (Figure 3) (Frost et al., 1997). It turns out that PAK, which is a Cdc42 and Rac target, can phosphorylate MEK1 on Ser298 in a region that mediates the interaction of MEK1 with Raf. More recent work has introduced more complexity to this cross-talk mechanism, since PAK can also phosphorylate Raf on Ser338, and this appears to be essential for integrin-mediated activation of ERK in COS7 cells (King et al., 1998; Chaudhary et al., 2000). Activation of PAK in these cells is dependent upon PI 3-kinase-dependent activation of Rac (King et al., 1998), though in Rat-1 cells, PAK activation occurs through a PI 3-kinase-mediated activation of another ser/thr kinase, Akt and independently of Rac (Figure 3) (Tang et al., 2000).

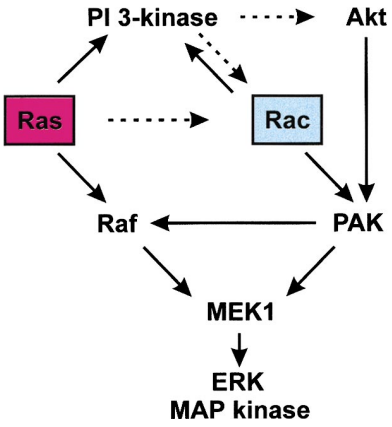

Figure 3. Signal Convergence Downstream of Ras and Rac

The Ras-Raf-MEK1-ERK MAP kinase cascade can be influenced by PAK-dependent phosphorylation of either Raf or MEK1. PAK can be activated through a direct interaction with Rac or independent of Rac via Akt. It seems unlikely that all these possibilities exist in the same cell at the same time and the pathway operating in any particular biological process will need to be defined. Solid lines represent direct protein:protein interactions and dotted lines represent the presence of intermediary steps.

PI 3-kinase acts through its lipid product, PIP3, to mediate activation of Rac downstream of many tyrosine kinase receptors (Nobes et al., 1995). As discussed above, it is thought that the lipid interacts with some PH domains found in GEFs and that this leads to relocalization and/or activation of Rac exchange factors. In addition, PI 3-kinase can itself interact directly with small GTPases. The p110 catalytic subunit interacts with the GTP-bound form of Ras, while the p85 regulatory subunit interacts with Cdc42 or Rac through a bcr GAP-homology domain (Kodaki et al., 1994; Zheng et al., 1994). Both interactions have been reported to lead to increased lipid kinase activity. The relationship between small GTPases and PI 3-kinase is, therefore, complicated since the latter can apparently act both upstream and downstream of Ras and Rac (Nobes et al., 1995; Keely et al., 1997; Sander et al., 1998). The synergy between Ras and Cdc42/Rac in regulating PI 3-kinase has not been explored. Overall, as illustrated in Figure 3, the potential complexity of cross-talk between Ras- and Rac-dependent signals is certainly striking. It is likely that only a subset of these connections are utilized in a given cell type in reponse to a specific agonist.

Another example of a molecule with the potential to connect Ras and Rho GTPase signaling pathways is p120RasGAP. This GTPase activating protein downregulates Ras, but speculation has been rife for fifteen years that it might also be a Ras effector and, through its N-terminal SH2 and SH3 domains, mediate some of the cellular effects of Ras. This issue has not been resolved, but it has been shown that p120RasGAP forms a complex in cells with p190RhoGAP. Furthermore, expression of the isolated N terminus of p120RasGAP in fibroblasts

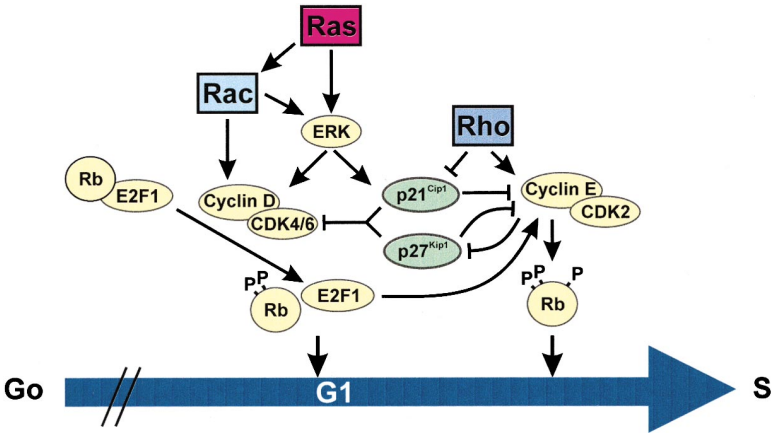

Figure 4. Influence of Ras and Rho Proteins on G1 Progression

Cell cycle regulatory proteins targeted by Ras, Rac, and Rho signal transduction pathways are shown. Cyclin D is a major target for Ras and Rac signaling, whereas Rho activity contributes primarily to the downregulation of the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ (see text for details).

efficiently inhibits Rho signaling (as judged by loss of stress fibers and focal adhesions), suggesting that p120RasGAP can potentiate the ability of p190RhoGAP to act as a downregulator of Rho (McGlade et al., 1993). This appears, therefore, to be a case of convergent interference of GTPase pathways. One other group has, however, raised some doubts about this simple idea; on the basis of their finding using neutralizing antibodies against the N-terminal region of p120RasGAP, they agree that p120RasGAP is a Ras target, but they suggest it promotes rather than inhibits the assembly of focal adhesions and stress fibers (Leblanc et al., 1998). Clearly, this cannot be through inhibition of Rho activity.

Finally, phospholipase D1 (PLD1) is an enzyme that can be regulated by direct interaction with a variety of molecules including small GTPases and lipids (Exton, 1999). Its activity is often upregulated in response to growth factors and in cells transformed by oncogenes such as v-Src and v-Raf. The high levels of PLD1 activity in v-Src-transformed cells have been reported to be dependent on Ral, and recently it has been shown that PLD1 upregulation in v-Raf transformed cells is both Ral and Rho dependent (Jiang et al., 1995; Frankel et al., 1999). Ral and Rho can each interact directly with PLD1, but whether they bind to the same or distinct sites on the molecule is not known (Luo et al., 1997).

Clearly, we have only scratched the surface in deciphering the molecular links between Ras and Rho GTPases. Although we have an increasingly good understanding of the genetics and biochemistry of these GTPases, the mechanisms underlying their combinatorial interactions are still relatively poorly defined. Given the potential complexity of these interactions, future work needs to focus on developing molecular tools to investigate how communication between Ras and Rho GTPases is orchestrated spatially and temporally.

Biological Contexts for Cross-Talk

The existence of signaling mechanisms that link Ras and Rho GTPases argues for the physiological importance of cross-talk between these GTPases. This is reflected by the growing number of examples of biological responses that depend on the coordinated activation of members of both protein families, the best characterized of which are discussed in the sections that follow.

Cell Proliferation

Cell cycle progression depends on cyclin-dependent protein kinases whose activity increases and decreases periodically during the growth and division of cells (Sherr, 1996). A critical role for Ras in cell cycle progression was suggested by early observations that the disruption of Ras function, either by microinjection of neutralizing anti-Ras antibodies or by expression of dominant interfering Ras, prevents mitogen-induced passage through G1 and entry into S phase (Mulcahy et al., 1985). A series of recent studies has begun to elucidate the biochemical principles underlying cell cycle control by Ras. An important new concept to emerge from these studies is that the proliferative effects of Ras depend, at least under some circumstances, on signaling inputs from its relatives Rac and Rho. Potential points of intersection between these signals and the cell cycle machinery are illustrated in Figure 4.

In quiescent rat embryo fibroblasts, expression of constitutively activated forms of Rac and Raf is sufficient to stimulate G1/S transition indicating that Rac- and Raf-dependent signals act synergistically to regulate cell cycle progression (Joneson and Bar-Sagi, 1998). The Raf-MEK-ERK cascade is responsible for the activation of CDK4 and CDK6 at the early stage of G1 through mechanisms involving primarily the transcriptional induction of cyclin D1 and posttranslational regulation of the assembly of cyclin D-CDK 4/6 complexes (Cheng et al., 1998; Kerkhoff and Rapp 1988). The principal biochemical activity of these complexes is to phosphorylate the Rb protein, causing the activation of E2F transcription factors that regulate the transcription of genes required for G1/S transition (Figure 4). The loss of Rb circumvents the requirement for Ras in cell proliferation (Mittnacht et al., 1997; Peeper et al., 1997), indicating that cyclin D1 is indeed a critical target of the mitogenic Ras signaling cascade.

The biochemical nature of the signals that couple Rac to the cell cycle machinery has so far remained elusive. Activated Rac induces cyclin D1 accumulation and dominant negative Rac moderately interferes with Ras-induced cyclin D1 expression (Westwick et al., 1997; Gille and Downward 1999). The ability of Rac to stimulate transcription from the cyclin D1 promoter correlates with its ability to activate PAK and requires the transcription factor NF-κB (Joyce et al. 1999). The collaborative action

of Rac and the ERK cascade in the induction of cyclin D1 expression could provide a mechanism to achieve sufficient levels of cyclin D1 expression. Additionally, Rac activity might be required to sustain cyclin D1 expression during progression through G1 under conditions where activation of the ERK cascade is transient. Until tested directly, these ideas remain entirely speculative.

The capacity of Rac to cooperate with Raf in the induction of S phase entry depends on a short region encompassing amino acids 124–135 of Rac, referred to as the insert region (Freeman et al., 1996). This region has been shown to be necessary for Rac-mediated activation of NADPH oxidase in phagocytes (Joseph and Pick, 1995; Freeman et al., 1996) and generation of superoxide in fibroblasts (Joneson and Bar-Sagi, 1998). Inhibition of superoxide production interferes with the mitogenic activity of Rac (Joneson and Bar-Sagi, 1998). Furthermore, superoxide generation has also been implicated in the proliferative effects of Ras (Irani et al., 1997). In this context, it is of interest to note that in airway smooth muscle cells, Rac-mediated cyclin D1 promoter activation requires the production of reactive oxygen species (Page et al., 1999). Altogether, while these observations identify reactive oxygen species as potential intermediates linking Rac and the cell cycle, a molecular understanding of this link awaits the identification of cellular targets of Rac-mediated superoxide production and characterization of the enzymatic oxidase activity present in non-phagocytic cells.

In some cell types, activation of the Raf-ERK MAP kinase cascade by overexpression of activated Ras or Raf leads to cell cycle arrest due to the induction of the CDK inhibitor $p21^{Cip1}$ (Figure 4) (Sewing et al., 1997; Woods et al., 1997). It has been shown that Rho suppresses the induction of $p21^{Cip1}$, thus enabling Ras to stimulate cell cycle progression (Olson et al., 1998). Cells lacking $p21^{Cip1}$ do not require Rho signaling for Ras-induced S-phase entry, indicating that the major contribution of Rho to the mitogenic effects of Ras is to interfere with the induction of $p21^{Cip1}$. The antagonistic effect of Rho on Ras-induced increase in $p21^{Cip1}$ levels is mediated, at least in part, by the suppression of $p21^{Cip1}$ transcription (Olson et al., 1998), however, the signaling pathways utilized by Rho to prevent induction of $p21^{Cip1}$ are still unknown. Another point of regulation of cell cycle progression by Rho is the induction of degradation of the CDK inhibitor $p27^{Kip1}$ through the stimulation of cyclin E/CDK2 activity (Weber et al., 1997; Hu et al., 1999). Thus, Rho may contribute to cell cycle progression by engaging two distinct mechanisms for the removal of cell cycle inhibitory activities.

Oncogenic Transformation

Evidence that the transforming activity of Ras requires functional Rac and Rho proteins has been provided by studies showing that dominant negative mutants of Rac and Rho inhibit Ras-induced transformation, and constitutively activated mutants of Rac and Rho cooperate with a constitutively activated Raf mutant in the induction of cellular transformation (Khosravi-Far et al. 1995; Qiu et al., 1995a, 1995b). Given the above described synergistic interactions of Ras and Rho GTPases in promoting cell cycle progression, this cooperativity is likely to contribute to the increase in the proliferative capacity displayed by Ras-transformed cells. Another important step in the process leading to oncogenic transformation is the activation of the cell survival machinery. The transcription factor NF-κB has been implicated in the induction of anti-apoptotic genes (Baichwal and Baeuerle 1997). Oncogenic Ras activates NF-κB-dependent transcription, and NF-κB activity is required for Ras-mediated transformation (Finco et al., 1997; Mayo et al., 1997). Specifically, Ras-induced activation of NF-κB appears to be critical for suppressing oncogene-induced apoptosis (Mayo et al., 1997). Both Rac and Rho have been shown to stimulate NF-κB-dependent transcription, albeit by different mechanisms (Sulciner et al., 1996; Perona et al., 1997; Montaner et al. 1998). This activity might explain, at least in part, their role in oncogenic Ras transformation.

Studies using Rho effector loop mutations that selectively disrupt effector interactions have demonstrated that the transforming potential of Rho correlates not with its capacity to stimulate serum response factor (SRF)-dependent transcription, but with its ability to bind Rho kinase/ROCK, an effector kinase involved in cytoskeletal reorganization (Leung et al., 1996; Sahai et al., 1998). Accordingly, the specific ROCK inhibitor Y-27632 interferes with Ras-mediated transformation, and constitutively active mutants of ROCK collaborate with activated Raf in transformation assays (Sahai et al., 1999). ROCK has been implicated in Rho-dependent assembly of focal adhesion complexes during integrin-induced cell adhesion (Amano et al., 1997; Ishizaki at al., 1997; Tominaga and Barber, 1998). Thus, it has been proposed that Rho activity might contribute to oncogenic transformation by mimicking signals that are generated by cell anchorage (Schwartz et al., 1996). However, this conclusion remains tentative in light of the apparently contradicting findings that interference with the Rho-ROCK pathway promotes Ras-induced transformation (Izawa et al., 1998).

Oncogenic transformation by Ras is characterized not only by enhanced growth rates but also by pronounced morphological changes resulting from alterations in the organization of the actin cytoskeleton and adhesive interactions. Epithelial cells transformed by oncogenic Ras acquire a mesenchymal phenotype which is associated with a decrease in cell–cell adhesion and an increase in focal adhesions and stress fibers (Zhong et al., 1997; Zondag et al., 2000). Since the assembly of focal adhesions and formation of stress fibers is stimulated by Rho, it has been proposed that the mesenchymal phenotype of Ras-transformed epithelial cells is due to the activation of Rho. In support of this idea are the observations that Ras-transformed epithelial cells display elevated levels of Rho activity and inhibitors of Rho partially restore the epithelial phenotype (Zhong et al., 1997). A recent insight into the mechanism by which oncogenic Ras might activate Rho has been provided by analyzing the functional interactions between Rac and Rho. In NIH 3T3 fibroblasts, Rac expression induces the downregulation of Rho activity, which in turn leads to the acquisition of an epithelial-like phenotype (Sander et al., 1999). In MDCK epithelial cells, oncogenic Ras downregulates Rac activity, which leads to the upregulation of Rho activity (Zondag et al., 2000). The downregulation of Rac activity results from a sustained activation

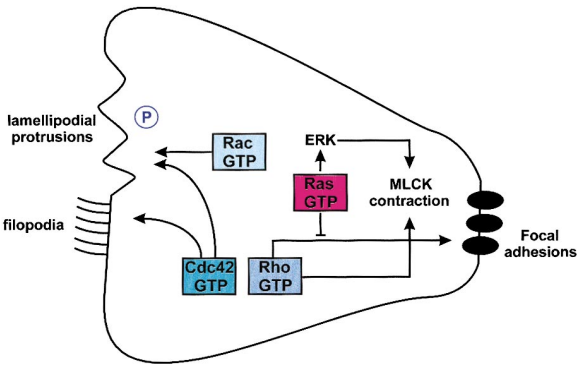

Figure 5. Cooperation of Ras and Rho GTPases in Cell Migration

Rac is required for the formation of protrusions at the front of the migrating cells and this is thought to provide the major driving force for movement. Cdc42 induces filopodia but their role in migration is unclear. In addition, Cdc42 provides a polarity signal (P) which ensures that Rac-induced protrusions are restricted to the leading edge. Migrating fibroblasts contain focal adhesions and these need to be disassembled at the rear of the cell. This might be achieved by Ras-dependent inhibition of Rho. Finally, contraction of the rear of the cell is thought to be required and a role for ERK-dependent phosphorylation of myosin light chain kinase (MLCK) has been reported to be involved.

of Raf/ERK signaling that causes the transcriptional repression of the Rac exchanger Tiam1. While it is still unclear how Rac downregulates Rho and to what extent the influence of oncogenic Ras on the relative activities of Rac and Rho is common to epithelial cells other than MDCK cells, it is reasonable to assume that cross-talk between Ras, Rac, and Rho contributes to the morphologic phenotype of Ras-transformed cells.

The Actin Cytoskeleton

The actin cytoskeleton plays an important role in defining cell shape and morphology and in orchestrating many of the dynamic aspects of cell behavior such as cell migration, axon guidance, phagocytosis, and cytokinesis. Cell migrations, often accompanied by changes in differentiated phenotype, are of great importance during embryonic development, but are also crucial in the adult during inflammatory and wound responses as well as tumor metastasis and invasion. There is now abundant evidence that Ras and Rho GTPases make distinct and cooperative contributions to these processes.

In mammalian cells, Rac is crucial for generating the actin-rich lamellipodial protrusions that are thought to be a major part of the driving force for movement (Ridley et al., 1992). In a macrophage chemotaxis assay and an epithelial cell scatter assay (where cells move as individuals), and in a fibroblast wound healing assay (where cells move as a sheet), inhibition of Rac completely prevents movement (Ridley et al., 1995; Allen et al., 1998; Nobes and Hall, 1999). Similarly, Rac is required for PDGF-induced migration of fibroblasts in a Boyden chamber assay, and for fibroblast invasion into a collagen matrix (Banyard et al., 2000). Cdc42 does not appear to be required for movement itself, but instead controls polarity signals that are required for directed migration (Figure 5) (Allen et al., 1998; Nobes and Hall, 1999). In the absence of Cdc42, macrophages, for example, no longer recognize a gradient of chemoattractant and instead move randomly (Allen et al., 1998).

The contribution of Rho to cell migration is less clear. In the macrophage chemotaxis assay, Rho is required for movement and it has been suggested that its activity might be restricted to the rear of the cell to generate the retraction forces (actomyosin-dependent) needed to pull the cell body along (Figure 5) (Allen et al., 1998). However, this attractive idea has not been directly demonstrated and the interpretation of these results is complicated by the fact that Rho is required for the adhesion of some cells to extracellular matrix. Partial inhibition of Rho actually speeds up cell migration in fibroblast wound healing assays, but complete Rho inhibition causes cell detachment (Nobes and Hall, 1999). Rho, acting through one of its targets, ROCK, promotes the invasion of a hepatoma cell line both in an in vivo and in vitro assay (Itoh et al., 1999), but whether this is due to an effect of Rho on the actin cytoskeleton or on gene transcription is not known.

Ras is known to make an essential contribution to cell migration in endothelial and fibroblast wound healing assays, in epithelial cell scatter assays, and in carcinoma cell Boyden chamber-type assays, but its role may be cell type dependent (Fox et al., 1994; Ridley et al., 1995; Klemke et al., 1998; Nobes and Hall, 1999). In endothelial cells, the role of Ras is in part accounted for by the ERK MAP kinase pathway. However, this cannot be the case in the fibroblast wound healing assay, where ERK does not play a significant role. In these cells, Ras appears to be involved in focal adhesion turnover, since in the absence of these adhesive structures, the cells do not require Ras for movement (Nobes and Hall, 1999). As focal adhesion assembly is controlled by Rho, one interesting possibility is that Ras downregulates Rho in these cells (Figure 5). A good candidate for mediating this connection was introduced earlier—p120RasGAP. Motility assays done on fibroblasts lacking p120RasGAP appear to confirm that this protein, through its interaction with p190RhoGAP, plays a role in focal adhesion turnover (Kulkarni et al., 2000).

Experiments with transmigration assays using a Boyden chamber also point to an important role for both Ras and Rac in migration (Klemke et al., 1998). Ras, through the ERK MAP kinase pathway, leads to phosphorylation of myosin light chain kinase, which in turn phosphorylates myosin, causing increased contraction of actin:myosin filaments (Figure 5) (Cheresh et al., 1999). This may play an important role at the rear of migrating cells facilitating translocation of the cell body. Rac, on the other hand, is required for the formation of actin-dependent cell protrusions found at the leading edge (Figure 5). A key molecule in promoting the migration of cells may be p130CAS; anchorage dependent phosphorylation of this protein on tyrosine results in its association with an SH2/SH3-containing adaptor protein, Crk, and the recruitment of DOCK180, leading to Rac activation. ERK activation appears to occur independently of this signaling complex. Interestingly, although Rac and ERK have both been shown to be essential for the transmigration of pancreatic carcinoma cells, Ras itself appears not to be required (Klemke et al., 1998; Cheresh et al., 1999). In another study of growth factor–induced cell migration, Rac was shown to contribute to the activation of ERK by synergizing with Raf (Leng et al., 1999). Rac may, therefore, have at least two

distinct roles in cell migration, first, to regulate actin polymerization, leading to membrane protrusions, and second, to facilitate activation of ERK MAP kinase.

Closer examination of the assays used in these various experiments highlights some of the problems in interpreting migration studies. The wound healing experiments using endothelial cells, for example, were done over a period of 24 hr, where gene transcription and protein synthesis are required, while the fibroblast assays were carried out over a short, 6 hr time period; conditions where gene transcription is not required (Nobes and Hall, 1999). Invasion or Boyden chamber assays are also long term, and since both Ras and Rho GTPases can regulate gene transcription as well as the actin cytoskeleton, their contribution to the overall process is difficult to dissect. In cell scatter assays using MDCK cells, careful inspection of the cells reveals that Ras is essential for the early transcription-independent, morphological changes that precede cell migration (Ridley et al., 1995). Clearly, many biochemical details remain to be clarified, but there is little doubt that the coordinated activities of at least four small GTPases (Cdc42, Rac, Rho, and Ras) are required for cell migration in a variety of cell types.

The actin cytoskeleton is required for many other biological processes in addition to cell migration and Rho, Rac, and Cdc42 have each been shown to play an essential regulatory role during phagocytosis in macrophage cells. Cdc42 and Rac are both required for Fcγ receptor-mediated internalization of immunoglobulin opsonized microrganisms (Cox et al., 1997; Caron and Hall, 1998). Furthermore, phagocytosis through this receptor is accompanied by the activation of an NADPH oxidase, which generates superoxide radicals as part of the bacterial killing mechanism, and Rac is an allosteric regulator of this enzyme (Abo et al., 1991). Biochemically, the NADPH oxidase complex copurifies with Rap1 and while its exact role in enzyme activation is unclear, this suggests cooperativity between Rap1 and Rac in the generation of reactive oxygen species (Bokoch et al., 1991; Gabig et al., 1995).

Phagocytosis of complement opsonized particles occurs through the CR3 receptor in macrophages and is Rho, but not Cdc42 or Rac, dependent (Caron and Hall, 1998). The CR3 receptor is an integrin (αMβ2) and in resting macrophages it is found at the cell surface, but in an inactive state unable to recognize particles. Through a process of inside-out signaling, a variety of inflammatory mediators can induce activation of the integrin. It has recently been shown that three quite different agonists, LPS (lipopolysaccharide), TNFα, and PAF (platelet activating factor) each activate this receptor through a Rap1-dependent signal transduction pathway (Caron et al., 2000). In this case, Rap1 and Rho cooperate with each other during complement-mediated phagocytosis by regulating two sequential steps in the pathway, namely integrin activation and integrin internalization.

Mechanisms of Signal Integration

Irrespective of the biological setting, a major mechanistic challenge posed by the combinatorial utilization of Ras and Rho GTPases is how the multiple signals that they transmit are processed coordinately to give rise to a specific physiological response. Our understanding of the molecular makings of signal integrators is still in its early days, but a few common regulatory themes are beginning to emerge. Here, we shall discuss two cellular machineries which have been clearly implicated in the integration of Ras and Rho GTPase signals: the transcriptional machinery and the cell adhesion machinery.

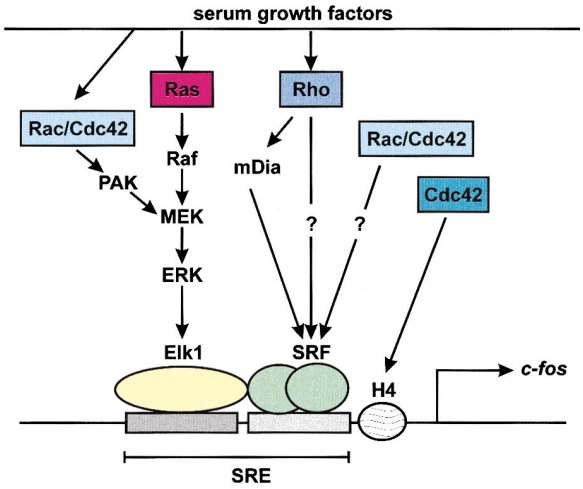

Figure 6. Transcriptional Activation of the *c-fos* SRE by Ras and Rho GTPases

An illustration of the transcription factors at the *c-fos* SRE that are targets for Ras and Rho signaling pathways. This model is based primarily on data derived from studies using transfected SRE or SRF reporters. Chromosomally integrated SRF reporters cannot be activated solely by Rho-dependent signals but require additional signals that can induce hyperacetylation of histone H4. These signals can be induced in part by Cdc42.

Transcriptional Activation of the Serum Response Element

The serum response element (SRE) is a conserved promoter element that mediates the induction of many cellular immediate early genes following mitogenic stimulation. The activity of the SRE is dependent on the binding of the ubiquitous transcription factor SRF (Norman et al., 1988). In the context of the *c-fos* SRE, SRF forms a ternary complex with members of the family of Ets domain protein, TCFs, and these interactions are required for the full regulatory activity of the SRE (Shaw et al. 1989). The SRF-TCF ternary complex is subject to regulation by converging and parallel signals from Ras and Rho GTPases, thus forming a molecular device for signal integration (Figure 6).

The Ras/Raf/ERK pathway regulates the *c-fos* SRE activity by targeting the TCF protein Elk-1. ERK phosphorylates Elk1 on its C-terminal activation domain, thereby potentiating the ability of Elk1 to activate transcription (Marais et al., 1993). Rac and Cdc42 also regulate Elk1 through a similar mechanism involving ERK-dependent phosphorylation. The activation of ERK by Rac and Cdc42 occurs through PAK-mediated phosphorylation of MEK, and PAK activity is required for the full stimulation of Elk1-dependent transcription by serum growth factors (Frost et al., 1997). Thus, the activation of Elk1 by ERK MAP kinase constitutes a point

of convergence between signaling inputs from Ras and Rho GTPases. There is growing awareness that the extent and duration of ERK activation can dictate the nature of the biological response. For example, in PC12 cells, transient activation of the ERK pathway stimulates cell proliferation, whereas persistent ERK activation leads to neuronal differentiation (Dikic et al., 1994; Traverse et al., 1994). In mesenchymal cells, differences in the extent of ERK activation can lead to opposing effects on cell proliferation with low levels of ERK activation promoting cell cycle progression and high levels promoting cell cycle arrest (Sewing et al., 1997; Woods et al., 1997). The cooperative effects of Ras, Rac, and Cdc42 on Elk1 activation might provide a mechanism for the specification of the biological outcome in response to different levels of ERK MAP kinase signaling.

Although mutants of the *c-fos* SRE that are defective in Elk1 binding can no longer respond to the Ras-Raf-ERK pathway, they retain the ability to be activated by serum, indicating that SRF activity can be controlled by extracellular signals independently of TCFs (Hill et al., 1995).The Elk1-independent transcriptional activation of SRF in response to serum requires functional RhoA, and the Rho effector mDia has been identified as an essential component of the signaling pathway utilized by Rho to activate SRF (Figure 6) (Hill et al., 1995; Sotiropoulos et al., 1999, Tominaga et al., 2000). Activated forms of Rac and Cdc42 can also stimulate the transcriptional activity of SRF, but this occurs independently of Rho, suggesting that Rho GTPases can target SRF by at least two distinct signaling pathways (Hill et al., 1995). One further complexity to this regulatory circuitry is that whereas Rho-dependent signals are sufficient for the activation of transfected SRF reporters, chromosomally integrated SRF reporters cannot be activated by Rho alone but require additional signals that lead to histone H4 hyperacetylation (Alberts et al., 1998). These signals can be delivered by Cdc42 in part through the activation of the SAPK/JNK pathway. A recent study has implicated actin dynamics in the regulation of SRF activation by Rho GTPases; however, this mode of regulation is restricted to a subset of SRF target genes and does not contribute to the activation of the *c-fos* SRE (Sotiropoulos et al., 1999).

Collectively, it appears that the signaling pathways activated by Rho and Ras converge at the *c-fos* SRE via different transcription factors; Ras-dependent signals target Elk1 whereas Rho-dependent signals target SRF (Figure 6). In principle, such molecular arrangement provides an effective mechanism for conferring specificity to the transcriptional response. For example, stimuli that can simultaneously activate Ras and Rho signaling might elicit one type of transcriptional response, while stimuli that selectively activate Ras or Rho signaling would give rise to a different transcriptional response. In support of this concept, it has been shown that the optimal activation of the *c-fos* gene in response to various stimuli requires both functional Ras and Rho (Hill et al. 1995). The *c-fos* SRE is not the only transcriptional element to be regulated by Ras and Rho GTPases, and additional examples include the transcriptional activation of the IL-2 and NF-κB genes (Finco et al., 1997; Perona et al., 1997; Henning and Cantrell 1998; Penninger and Crabtree 1999) underscoring the importance of the transcriptional machinery in the integration of signal inputs generated by Ras and Rho GTPases.

Integrin-Mediated Matrix Adhesion

Most mammalian cells are dependent on adhesion to the extracellular matrix (ECM) for their growth, survival, and differentiation. It is well established that the effects of the ECM on cellular behavior are mediated by members of the integrin family of cell surface adhesion molecules. The binding of integrins to matrix proteins triggers a variety of intracellular signaling events, including the activation of Ras and Rho GTPases (outside-in signaling). Activated Ras and Rho GTPases in turn regulate the extracellular binding activity of integrins (inside-out signaling), primarily through their effects on the actin cytoskeleton. Many aspects of these bidirectional interactions have been addressed in several recent reviews (Howe et al., 1998; Giancotti and Ruoslahti, 1999; Schoenwaelder and Burridge, 1999). Cell attachment through integrins also modulates the signaling activities of Ras and Rho GTPases in response to serum growth factors by mechanisms that are just beginning to be uncovered. As discussed below, they reveal the foundations of a pathway by which the signaling outputs of Ras and Rho GTPases can be integrated in the context of mitogenic signaling.

In some cells, loss of matrix attachment results in the inhibition of serum-induced activation of the Ras-ERK cascade (Lin et al. 1997b; Renshaw et al., 1997). Although the mechanism by which this inhibition occurs is incompletely understood, it has been shown that the adhesion-dependent step is the activation of MEK by Raf (Renshaw et al., 1997). The synergistic interaction between cell adhesion and activation of the ERK cascade might explain, at least in part, why normal cell proliferation is dependent on integrin-mediated anchorage to ECM. Interestingly, the Ras-ERK pathway has been implicated in the suppression of integrin activation (Hughes et al., 1997), suggesting the existence of reciprocal regulation of adhesion and growth factor–dependent Ras signaling via feedback mechanisms.

The activation of Rac and Rho by serum growth factors is also regulated by integrin-mediated cell adhesion to ECM. In adherent cells, the LPA-mediated activation of Rho is transient, whereas in detached cells, LPA induces the sustained activation of Rho (Ren et al., 1999). Moreover, the steady-state levels of Rho activity are higher in suspended cells than in attached cells, implicating integrin-mediated adhesion in the negative regulation of serum-dependent Rho activation. Since Rho activity has been shown to positively regulate integrin-dependent cell adhesion (Chrzanowska-Wodnicka 1996; Amano et al., 1997), these opposing effects most likely reflect a negative feedback loop that enables dynamic changes in matrix-dependent adhesion during cell migration. The activation of Rac by serum is significantly attenuated in suspended cells in comparison with adherent cells (del Pozo et al., 2000). Furthermore, it has been demonstrated that the ability of Rac to activate its target PAK1 is inhibited in nonadherent cells through a mechanism involving the impairment of Rac recruitment to the cell membrane (del Pozo et al., 2000). Altogether, it appears that integrin-mediated cell adhesion can modulate simultaneously the efficiency of growth factor signaling through Ras and Rho GTPases. Thus, the state

of cell adhesion could serve as a sensing mechanism for the coordinated activation of Ras and Rho GTPases in response to spatial cues. Since this conclusion is based on studies performed using a limited number of cell types, the general relevance of this mechanism remains to be established.

Conclusions

The functional analysis of GTP binding proteins has most often led to the identification of a single, signal transduction pathway as being of particular importance; Gs regulates adenylyl cyclase and cAMP levels, Ras regulates the ERK/MAP kinase cascade and cell proliferation, and Rho GTPases regulate actin polymerization and the organization of the actin cytoskeleton. The identification of multiple target proteins for many of these GTPases (Rac has 12 so far) has, however, made this idea of simple linear pathways untenable and there is now little doubt that members of the Ras and Rho GTPase families each control multiple intracellular pathways. Some of the potential biological implications of this have been most clearly revealed in *S. cerevisiae*, where Rho1p, for example, coordinately controls three distinct biochemical pathways, each of which contributes to the growth of a new bud during cell division. Similarly, in mammalian cells, the ability of Ras to regulate several pathways, not just ERK MAP kinase, explains why it is so efficient at inducing a malignant phenotype.

We have focused this review specifically on examples of cross-talk between Ras and Rho GTPases in animal cells. While the biochemical details by which this is achieved are still poorly understood, there is much experimental work that points to the importance of combinatorial activities controlled by these two families in promoting complex biological responses such as cell proliferation, cell transformation, and cell migration. Coordinated regulation of nucleotide exchange factors ensures that distinct subsets of GTPases are activated in response to a given agonist, while the specific recruitment of GAPs can provide a mechanism by which one GTPase leads to inactivation of another. The multiple pathways downstream of Ras and Rho GTPases can act in parallel to provide distinct biochemical activities and they can act synergistically such that activation of one pathway is dependent on the activation of another. So far, examples of each of these scenarios have been observed, although this is probably only the tip of the iceberg. Only as we learn more about the cellular roles of the twenty or so GTPases that constitute the mammalian Ras and Rho families will the biological versatility of these regulatory molecules be fully appreciated.

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