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Rac and Cdc42 Induce Actin Polymerization and G1 Cell Cycle Progression Independently of $p65^{PAK}$ and the JNK/SAPK MAP Kinase Cascade

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Summary

Rac and Cdc42 regulate a variety of responses in mammalian cells including formation of lamellipodia and filopodia, activation of the JNK MAP kinase cascade, and induction of G1 cell cycle progression. Rac is also one of the downstream targets required for Ras-induced malignant transformation. Rac and Cdc42 containing a Y40C effector site substitution no longer intact with the Ser/Thr kinase $p65^{PAK}$ and are unable to activate the JNK MAP kinase pathway. However, they still induce cytoskeletal changes and G1 cell cycle progression. Rac containing an F37A effector site substitution, on the other hand, no longer interacts with the Ser/Thr kinase $p160^{ROCK}$ and is unable to induce lamellipodia or G1 progression. We conclude that Rac and Cdc42 control MAP kinase pathways and actin cytoskeleton organization independently through distinct downstream targets.

Introduction

The Rho family of GTP-binding proteins has been implicated in the regulation of a wide range of biological processes, including cell motility, cell adhesion, cytokinesis, cell morphology, and cell growth (Hall, 1994). To date six distinct members of the Rho family have been found in mammalian cells: Rho (A, B, C isoforms), Rac (1, 2 isoforms), Cdc42 (Cdc42Hs, G25K isoforms), RhoG, RhoE, and TC10 (Hall, 1994; Foster et al., 1996), and one additional member, RhoL, has been identified in Drosophila (Murphy and Montell, 1996). Saccharomyces cerevisiae has five Rho-related proteins, Rho1, 2, 3, and 4 and Cdc42 (Chant and Stowers, 1995). Each of these small, monomeric GTPases is thought to act as a molecular switch to control intracellular signal transduction pathways. Rho GTPases exist in either an inactive (GDP-bound) or an active (GTP-bound) conformation and regulatory proteins that control this GDP/GTP cycle include over 15 (the DH family) distinct guanine nucleotide exchange factors (GEFs), around 10 GTPase-activating proteins (GAPs) and at least two guanine nucleotide dissociation inhibitors (GDIs) (Ueda et al., 1990; Lamarche and Hall, 1994; Cerione and Zheng, 1996).

A major function of Rho, Rac, and Cdc42 is to control the formation of polymerized actin structures and the assembly of associated integrin complexes. In Swiss 3T3 fibroblasts, activation of Rho by extracellular factors such as lysophosphatidic acid or bombesin leads to the formation of actin stress fibers and focal adhesion complexes, whereas activation of Rac by PDGF, EGF, or insulin leads to the formation of an actin meshwork at the cell periphery producing lamellipodia and membrane ruffles (Ridley and Hall, 1992; Ridley et al., 1992). Cdc42 activation is triggered by bradykinin and this leads to the formation of filopodial protrusions at the cell periphery (Kozma et al., 1995; Nobes and Hall, 1995). Rac and Cdc42 also induce the assembly of integrin complexes associated with the polymerized actin (Hotchin and Hall, 1995; Nobes and Hall, 1995). One further feature of these GTPase-controlled pathways is the cross-talk between them, such that Cdc42 activation leads to subsequent activation of Rac, which in turn leads to activation of Rho (Kozma et al., 1995; Nobes and Hall, 1995). These results have led to the suggestion that Rho, Rac and Cdc42 are likely to play a key role in regulating cell motility and cell adhesion in response to extracellular stimuli (Hall, 1994).

The role of these GTPases has been examined in many other cell types. In lymphoid cells for example, Rho is required for integrin-mediated cell aggregation; in the MDCK epithelial cell line, Rac is required for HGF-induced motility, and in helper T cells, Cdc42 controls cell polarity toward antigen-presenting target cells (Morii et al., 1992; Tominaga et al., 1993; Ridley et al., 1995; Stowers et al., 1995; Laudanna et al., 1996). The role of Rac in neuronal development has been explored using transgenic mice, where expression of constitutively active Rac in Purkinje cells has a differential effect on axons, dendritic trunks and spines (Luo et al., 1996). A genetic analysis in Drosophila has revealed a role for Cdc42 and Rac1 in controlling cell shape changes during wing disc development and the assembly of actin at adherens junction of the wing disc epithelium, respectively (Eaton et al., 1995). Cdc42, Rac, and RhoL also appear to play distinct roles in Drosophila oogenesis; constitutive activation of RhoL leads to breakdown of subcortical actin in nurse cells and disruption of nurse cell–follicle cell contacts, whereas inhibition of Rac blocks the migration of border cells (Murphy and Montell, 1996).

There is growing evidence that members of the Rho GTPase family also play a critical role in cell growth, in particular during G1 of the cell cycle. Inhibition of Rho by C3 transferase was first reported to block growing cells in G1 and more recently it has been shown that microinjection of constitutively activated Rho, Rac, or Cdc42 induces G1 progression in quiescent Swiss 3T3 fibroblasts leading to DNA synthesis (Yamamoto et al.,

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1993; Olson et al., 1995). Rac and Rho are activated by oncogenic Ras in fibroblasts and there is now compelling evidence that they are essential components of Ras-induced malignant transformation (Qiu et al., 1995; Khosravi-Far et al., 1995; Joneson et al., 1996). Taken together, these experiments support a role for Rho, Rac, and Cdc42 in the control of signaling pathways that regulate gene transcription and that are required for G1 progression. In apparent agreement with this, several groups have reported that Rac and Cdc42, but not Rho, regulate the c-Jun N-terminal or stress-activated MAP kinase (JNK/SAPK) and the p38/HOG MAP kinase cascades (Bagrodia et al., 1995; Coso et al., 1995; Minden et al., 1995; Olson et al., 1995; Zhang et al., 1995). Rho, on the other hand is required for serum-induced activation of the transcription factor SRF (serum response factor), which interacts with the serum response element found in many growth factor–regulated promoters (Hill et al., 1995).

Over the past few years, much effort has been put into identifying the downstream target molecules of Rac and Cdc42. $p120^{ACK}$, a nonreceptor tyrosine kinase, was first identified as a target for Cdc42 and interacts specifically with the active, GTP-bound conformation of the GTPase (Manser et al., 1993). Subsequently, the same group identified a ser/thr protein kinase, $p65^{PAK}$, that interacts with the active forms of both Rac and Cdc42, but not Rho (Manser et al., 1994). The kinase activity of $p65^{PAK}$ is greatly enhanced by the interaction with Rac-GTP or Cdc42-GTP, strongly suggesting that it is a physiological target. Analysis of the GTPase binding sites of $p120^{ACK}$, $p65^{PAK}$, and the related yeast protein Ste20 has led to the identification of a minimal region of 16 amino acids required for Cdc42 or Rac-interactive binding (CRIB motif). More than 25 proteins from a wide variety of eukaryotic species and containing a similar motif were identified in a search of the GenBank data base, and a number of these have been shown to interact with Cdc42 or Rac in vitro in a GTP-dependent manner (Burbelo et al., 1995). These include an uncharacterized mammalian protein MSE55, a novel mammalian kinase MLK3, a C. elegans protein encoded by F09F7.5, and a tyrosine kinase, DPR2, from Drosophila. The product of the human Wiskott-Aldrich syndrome gene, WASP, also contains a CRIB motif, and this protein was isolated independently in a yeast two-hybrid screen using Cdc42 as bait and by column chromatography (Aspenström et al., 1996; Symons et al., 1996). In phagocytic cells, Rac regulates the activity of a multimeric, membrane-associated enzyme, the NADPH oxidase (Abo et al., 1991). A cytoplasmic-derived component of this complex, $p67_{PHOX}$ has been identified as the target for Rac in this activity (Diekmann et al., 1994). To date, $p67_{PHOX}$ is the only non-CRIB motif containing protein that has been found to interact directly with mammalian Rac.

To dissect the role of the various candidate targets, we have analyzed the consequences of introducing selected effector site amino acid substitutions in Rac and Cdc42; an approach first described to analyze Ras targets (White et al., 1995). We report here that a Y40C substitution in Rac and Cdc42 destroys the interaction with all CRIB motif–containing proteins and the GTPases are no longer able to activate $p65^{PAK}$ or the JNK/SAPK MAP kinase cascade. Despite this, Y40C-substituted Rac and Cdc42 trigger the assembly of lamellipodia and filopodia, respectively, and both are still able to induce DNA synthesis when injected into quiescent fibroblasts.

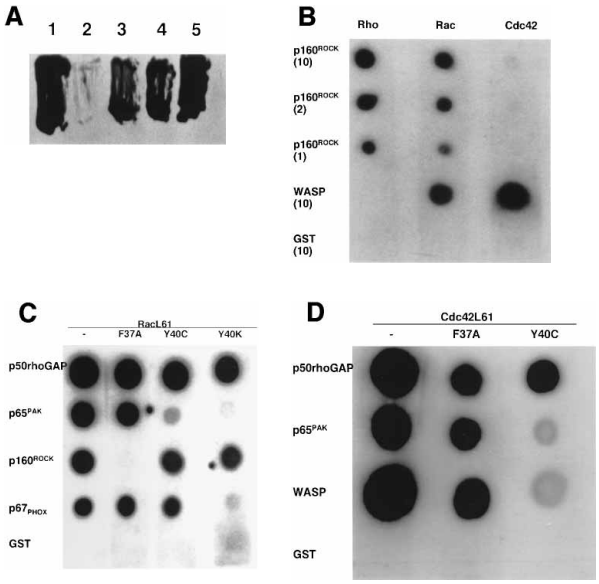

Figure 1. Interaction of Rac and Cdc42 with Candidate Target Proteins

(A) Interaction of $p160^{ROCK}$ with RhoL63, RacL61, and Cdc42L61 in the yeast two-hybrid system. Yeast strains containing integrated GAL4DB-plasmids with: (1 and 2) R-RasL87; (3) RhoL63; (4) RacL61; (5) Cdc42L61 were transformed with pACTII-$p160^{ROCK}$(aa 868–1030) (2–5) or pACTII-Raf-1 (1), and allowed to grow for two days at 30°C on SC-Trp-Leu-His plates. Colonies were restreaked on SC-Trp-Leu-His containing 50 mM 3-amino-1,2,4-triazole and allowed to grow for two days at 30°C. (B) GST-$p160^{ROCK}$ (aa 868–1030) (10 μg, 2 μg, or 1 μg); GST-WASP (aa 201–321) (10 μg) and GST protein (10 μg) were spotted onto a nitrocellulose filter in a volume of 5 μl and incubated with 0.1 μg of [γ-^{32}P]-GTP-loaded wild-type Rho, Rac, or Cdc42. (C) 10 μg of GST, GST-p50RhoGAP (aa 198–439), GST-$p65^{PAK}$, GST-$p160^{ROCK}$ (aa 868–1030), and GST-$p67_{PHOX}$ (aa 1–199) were spotted onto a nitrocellulose membrane and incubated with 0.1 μg of [γ-^{32}P]-GTP-loaded RacL61, RacL61A37, RacL61C40 or RacL61K40. (D) 10 μg of GST, GST-p50rhoGAP (aa 198–439), GST-$p65^{PAK}$ and GST-WASP (aa 201–321) were spotted on a nitrocellulose filter and incubated with 0.1 μg of [γ^{32}P]-GTP-loaded Cdc42L61, Cdc42L61A37 and Cdc42L61C40. GTPase interactions were visualized by autoradiography.

Results

Identification of an Additional Candidate Target Protein for Rac

Using a yeast two-hybrid screen of a human HeLa cDNA library with constitutively active RhoL63 as bait, we have isolated a clone that on sequence analysis corresponds to amino acids (aa) 868–1030 of the ser/thr kinase, $p160^{ROCK}$ (Ishizaki et al., 1996). $p160^{ROCK}$ also interacts with constitutively active, RacL61 and with Cdc42L61 in yeast, but not with constitutively activated R-ras (see Figure 1A). To confirm these interactions in vitro, we have expressed this region of $p160^{ROCK}$ as a GST fusion protein in E. coli. The protein was spotted onto nitrocellulose filters and its ability to bind the different [γ-^{32}P]GTP-bound GTPases assessed in a dot blot assay

(Diekmann et al., 1995). Under these conditions, $p160^{ROCK}$ interacts strongly with RhoL63, RacL61, and Cdc42L61 (data not shown), but as shown in Figure 1B when the wild-type GTPases are used, Rho and Rac, but not Cdc42, interact strongly with $p160^{ROCK}$. We estimate that the interaction with Rac is approximately 2-fold less than with Rho in this assay. We conclude that $p160^{ROCK}$ is a candidate target for Rho and Rac but not Cdc42. $p160^{ROCK}$ does not contain a CRIB motif, nor does it show any homology to $p67_{PHOX}$.

Interaction of Rac and Cdc42 with Target Proteins

To dissect the role of the different Rac and Cdc42 target proteins, we have introduced single amino acid substitutions into the effector loop of both GTPases. Based on previous work with the Ras effector region (White et al., 1995; Joneson et al., 1996), the following substitutions, F37A, Y40C, and Y40K, were introduced first into the constitutively active RacL61 to produce RacL61A37, RacL61C40, and RacL61K40. The proteins were expressed in E. coli and their interaction with target proteins assessed in a dot blot assay. As can be seen in Figure 1C, the Y40K substitution severely diminishes the interaction of Rac with the murine $p65^{PAK}$ kinase and with $p67_{PHOX}$, but has no effect on interaction with $p160^{ROCK}$. Interestingly, Y40C blocks interaction with $p65^{PAK}$, but not with $p67_{PHOX}$ or $p160^{ROCK}$. Finally, the F37A substitution eliminates interaction with $p160^{ROCK}$, but has no effect on $p65^{PAK}$ or $p67_{PHOX}$ binding. p50RhoGAP binding is unaffected by these effector site substitutions and is used as an internal control.

F37A and Y40C substitutions were also introduced into Cdc42L61 to produce Cdc42L61A37 and Cdc42L61C40. As shown in Figure 1D, Y40C has a similar effect to that found with Rac; namely it prevents interaction with $p65^{PAK}$. In addition, the Y40C mutant does not interact with any other CRIB-containing protein tested, including WASP (Figure 1D), MSE55 (data not shown), and the C. elegans protein F09F7.5 (data not shown). We conclude that the Y40C substitution disrupts the interaction of Rac and Cdc42 with all CRIB-containing targets, whereas the F37A substitution has no effect on the interaction with CRIB proteins.

Activation of $p65^{PAK}$ Kinase and the Stress-Responsive SAPK/JNK Pathway by Rac and Cdc42

To determine whether the in vitro interaction of Rac and Cdc42 mutants with $p65^{PAK}$ reflects their ability to activate the kinase in vivo, we have used COS cell transfection assays. Wild-type, human $p65^{PAK}$ (Brown et al., 1996), and various Rac and Cdc42 GTPase mutants were introduced into a eukaryotic expression vector, pRK5, along with a myc epitope tag at their N-termini. Constructs were coexpressed in COS-1 cells for 48 hr, $p65^{PAK}$ immunoprecipitated with an anti-myc monoclonal antibody, and its ser/thr kinase activity assessed in vitro using myelin basic protein (MBP) as a substrate. A constitutively activated $p65^{PAK}$, $p65^{PAK}$L107F, was used to compare the extent of Rac/Cdc42 activation of the wild-type protein (Brown et al., 1996).

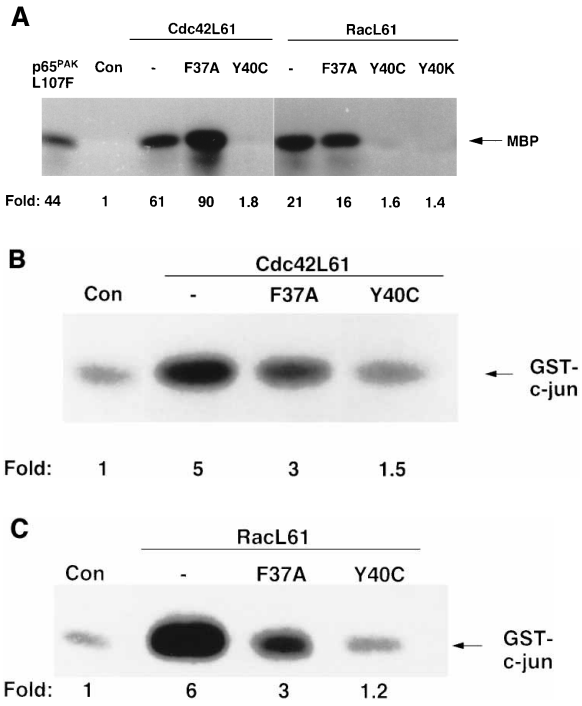

Figure 2. Activation of $p65^{PAK}$ and JNK/SAPK Cascade by Rac and Cdc42

(A) Cos-1 cells were transfected with 5 μg of pRK5myc-$p65^{PAK}$L107F or pRK5myc-$p65^{PAK}$ together with 1.5 μg of pRK5myc containing either no insert (Con), Cdc42L61 or RacL61 (−), or the indicated mutants. $p65^{PAK}$ activity was assessed by immunocomplex kinase assays using myelin basic protein (MBP) as a substrate and revealed by autoradiography. Lanes with the RacL61 expression vectors show a slightly longer exposure time compared to other lanes. The amount of $p65^{PAK}$ in each sample was determined by Western blotting using anti-myc antibody and shown to be similar. (B) Cos-1 cells were transfected with 5 μg of pCMV-FLAG-JNK1 and 1.5 μg of pRK5myc containing no insert (Con), Cdc42L61 (−), or the indicated mutants. (C) As described in (B) using 1.5 μg of the indicated RacL61 expression vectors. JNK1 activity was measured in immunocomplex kinase assays using GST-c-jun as substrate and revealed by autoradiography. JNK1 expression was determined by Western blotting using anti-FLAG antibody and shown to be similar in each sample. The fold activation of kinase activity was determined by phosphorimager quantitation and is indicated below each lane. For RacL61C40 and Cdc42L61C40, the fold activation of JNK activity was 1.24 ± 0.19 and 1.33 ± 0.15, respectively. These values are, within experimental error, indistinguishable from the control corresponding to JNK activity in the absence of the GTPases.

Figure 2A shows that RacL61 and Cdc42L61 are able to activate wild-type $p65^{PAK}$ to approximately the same level as found in the constitutively activated kinase. The F37A substitution has no significant inhibitory effect on the ability of either Rac or Cdc42 to stimulate kinase activity. In contrast, Y40C-substituted Rac and Cdc42, which do not interact with CRIB motif–containing proteins in vitro, are unable to activate $p65^{PAK}$ kinase when cotransfected into COS cells.

It has been shown that Rac and Cdc42 can stimulate kinase cascades leading to activation of the JNK/SAPK and the p38/HOG MAP kinases. (Coso et al., 1995; Minden et al., 1995; Olson et al., 1995). In addition, a constitutively activated form of $p65^{PAK}$ showed a stimulation of

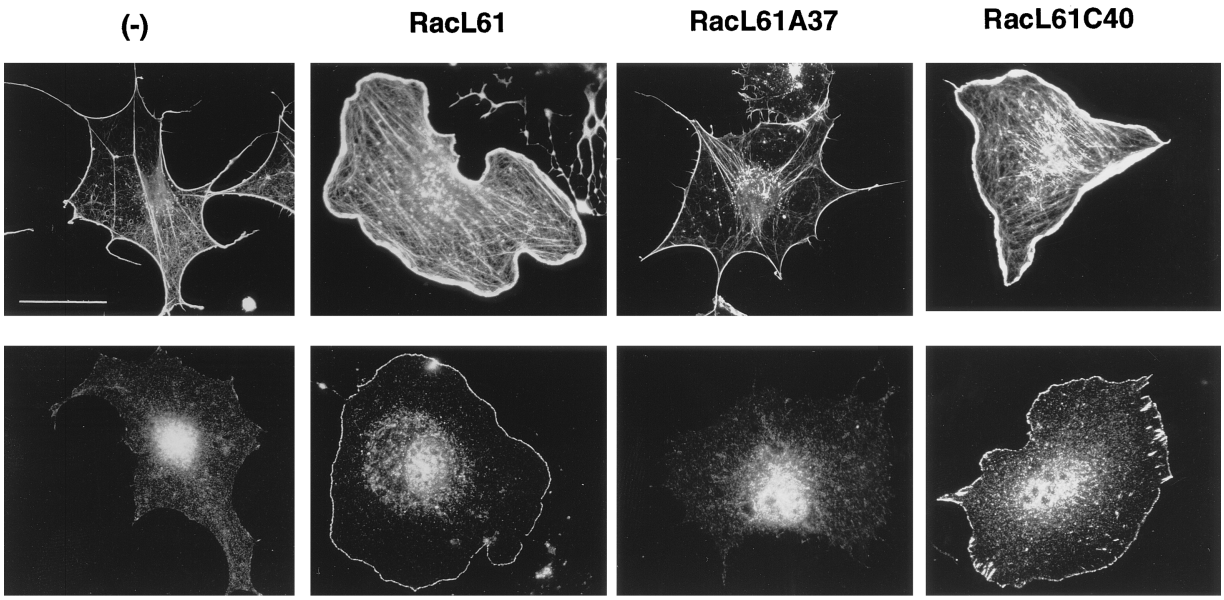

Figure 3. Lamellipodia and Integrin Complex Formation Induced by Rac

Serum-starved subconfluent Swiss 3T3 fibroblasts were fixed 2 hr after injection of pRK5myc encoding the indicated RacL61 proteins (0.2 mg/ml). In the bottom panel, Texas red–coupled dextran (5 mg/ml) was coinjected along with the indicated expression plasmids. In the top panel, actin filaments were visualized with fluorescently tagged phalloidin and injected cells were localized by costaining with an anti-myc antibody and by indirect immunofluorescence. In the bottom panel, vinculin was visualized by indirect immunofluorescence and injected cells were identified using the Texas red marker. Approximately 50 cells were microinjected per coverslip, and 2 hr after injection, 90% of the injected cells showed expression of the indicated GTPases and displayed the corresponding cellular effect. (−) indicates injected with empty vector. Scale bar, 30 μm.

JNK1 activity similar to that obtained with constitutively activated RacV12 (Bagrodia et al., 1995; Brown et al., 1996). It has been proposed, therefore, that $p65^{PAK}$ is the Rac and Cdc42 target protein responsible for activation of MAP kinase cascades. To test this idea, we have coexpressed a flag-tagged JNK1 expression plasmid with the various GTPase mutants described above, in COS-1 cells, and assayed JNK1 kinase activity in immunoprecipitates using recombinant c-jun as substrate. As shown in Figures 2B and 2C, F37A-substituted Rac and Cdc42 retained their ability to stimulate JNK1 activity, although stimulation was reproducibly 2-fold less than with nonsubstituted GTPases. The Y40C mutants were no longer able to stimulate JNK1 activity.

Rac-Induced Actin Polymerization and Integrin Complex Assembly

One of the major biological effects mediated by Rac is to control the polymerization of actin at the leading edge of the plasma membrane, leading to the extension of lamellipodia and the subsequent formation of membrane ruffles (Ridley and Hall, 1992). To explore the role of candidate Rac target proteins in this response, we have microinjected eukaryotic vectors (pRK5) encoding myc-tagged Rac into serum-starved subconfluent Swiss 3T3 cells and examined the distribution of filamentous actin. As shown in Figure 3 (top panel), 2 hr after microinjection of pRK5myc-RacL61 polymerized actin assembles at the leading edge of the plasma membrane to form lamellipodia; these are often seen folding back upon themselves to form ruffles. The Y40C mutant is as effective as RacL61 at inducing actin polymerization and lamellipodia formation. The F37A mutant, however, is totally unable to induce actin polymerization at the plasma membrane; no lamellipodia or membrane ruffles could be observed.

We previously reported that induction of actin polymerization at the cell margin by Rac is accompanied by the assembly of integrin-based focal complexes that can be visualized using an anti-vinculin antibody (Nobes and Hall, 1995). Figure 3 (bottom panel) shows that the Y40C mutant is able to induce vinculin-containing focal complexes at the cell margin, whereas the F37A mutant is not. We conclude that $p65^{PAK}$ and other CRIB motif-containing proteins do not mediate Rac-induced actin polymerization and focal complex formation leading to lamellipodia. The behavior of the F37A mutant suggests, however, that $p160^{ROCK}$ might be involved in these cellular effects.

$p65^{PAK}$ Does Not Mediate Filopodia Formation Induced by Cdc42

When microinjected into Swiss 3T3 cells, constitutively activated Cdc42 protein induces highly motile, actin-containing filopodial protrusions from the cell periphery (Kozma et al., 1995; Nobes and Hall 1995). Filopodia formation is accompanied by subsequent lamellipodial spreading in Swiss 3T3 cells, and since this can be inhibited by coinjection of dominant negative RacN17 protein, it has been concluded that activation of Cdc42 leads to activation of Rac (Kozma et al., 1995; Nobes and Hall 1995). To examine the effects of the various Cdc42 mutants on filopodia formation, we have coinjected expression vectors encoding myc-tagged Cdc42

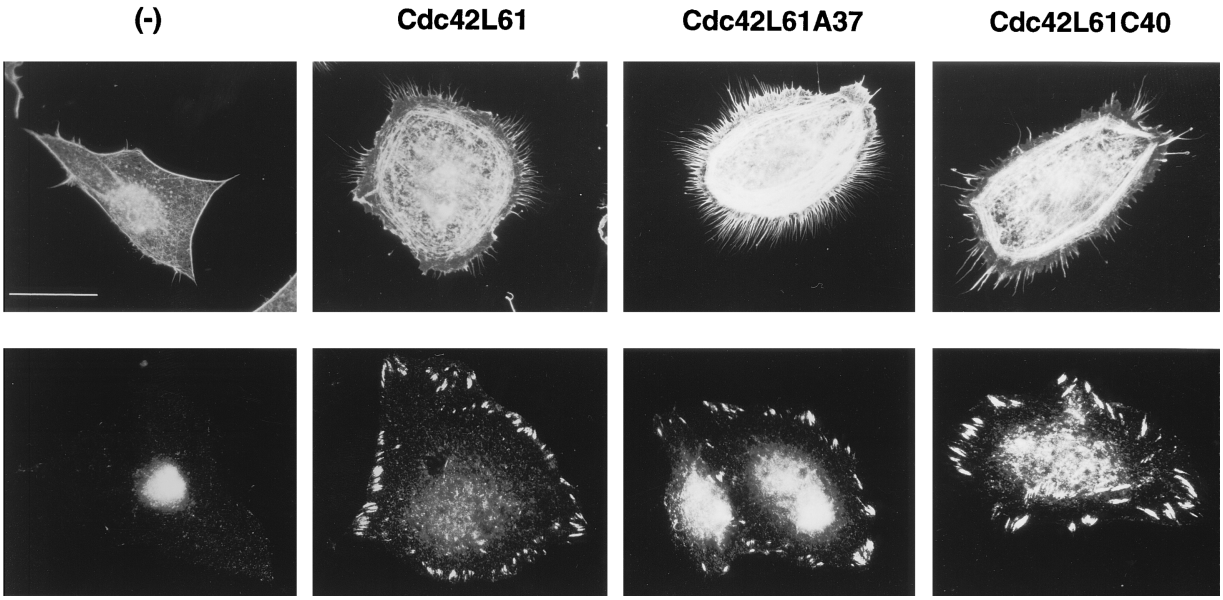

Figure 4. Filopodia and Integrin Complex Formation by Cdc42

Serum-starved subconfluent Swiss 3T3 cells were fixed 2 hr after coinjection of the indicated Cdc42L61 expression vectors and recombinant RacN17 protein (0.5 mg/ml). F-Actin (top panel) and vinculin (bottom panel) were visualized as in Figure 3.

with recombinant, dominant negative RacN17 protein into serum-starved, subconfluent Swiss 3T3 cells. As shown in Figure 4 (top panel), Cdc42L61 and both the F37A and Y40C mutants induce a "hairy" phenotype composed of fine filopodia (50–100 per cell) extending from the periphery of injected cells. Time-lapse cinematography confirmed that these structures resulted from membrane extensions (driven by actin polymerization) rather than membrane retraction. We have also noted a ring of actin bundles induced by Cdc42 under these experimental conditions.

The induction of filopodial actin filaments by Cdc42 is accompanied by the assembly of integrin complexes formed independently of those observed with Rac and Rho (Nobes and Hall, 1995). Figure 4 (bottom panel) reveals that both Cdc42 mutants are still able to induce focal complexes containing vinculin. We conclude that $p65^{PAK}$, WASP, and other CRIB motif–containing proteins do not mediate the downstream effects induced by Cdc42 leading to filopodia formation. Unlike Rac, however, the F37A substitution does not interfere with actin polymerization and focal complex assembly induced by Cdc42.

Cross-Talk between Cdc42, Rac, and Rho

A hierarchical relationship between the three GTPases has been suggested in Swiss 3T3 cells such that Cdc42 activates Rac and Rac activates Rho resulting in coordinated changes in actin reorganization (Ridley et al., 1992; Nobes and Hall, 1995). However, the biochemical mechanisms and the number of intermediate steps involved are currently not clear. To examine the consequences of the effector site mutations on this apparent cross-talk, we microinjected expression plasmids encoding Rac or Cdc42 into serum-starved, subconfluent Swiss 3T3 cells. As described above, 2 hr after microinjection of Rac, cells contain predominantly peripheral actin and small focal complexes (Figure 3). However, 4 hr after injection, cells also contain actin stress fibers and classical focal adhesions induced via activation of Rho. As shown in Figure 5 (top panel), the Y40C mutant of Rac also leads to activation of Rho, whereas the F37A mutant does not. When Cdc42L61 is microinjected in the absence of RacN17, cells are seen to contain predominantly lamellipodia and membrane ruffles due to activation of Rac (Figure 5, bottom panel) (Kozma et al., 1995; Nobes and Hall 1995). The Y40C mutant of Cdc42 also leads to activation of Rac; however, injection of the F37A mutant results in the formation of filopodia only, similar to the effects observed when Cdc42 was coinjected with RacN17 (compare Figure 4, top panel, and Figure 5, bottom panel). We conclude that CRIB-containing proteins are not involved in linking Cdc42 to Rac, or Rac to Rho, but that the F37A mutation destroys cross-talk between Cdc42, Rac, and Rho.

Stimulation of G1 Progression by Rac and Cdc42

Rac and Cdc42 have both been shown to play a role in mitogenic signaling; when microinjected into quiescent Swiss 3T3 cells, they stimulate cell cycle progression through G1 and DNA synthesis while dominant negative versions of Rac and Cdc42 block serum-induced DNA synthesis (Olson et al., 1995). Furthermore, Rac activation is thought to play a crucial role in Ras-induced malignant transformation and it can, itself, act as a weak oncogene (Qiu et al., 1995; Joneson et al., 1996). To examine the effects of the mutations, we have microinjected expression vectors encoding Rac and Cdc42 mutants into quiescent Swiss 3T3 cells and measured BrdU incorporation after 40 hr. Figure 6 shows that the Y40C-substituted Rac and both Y40C- and F37A-substituted

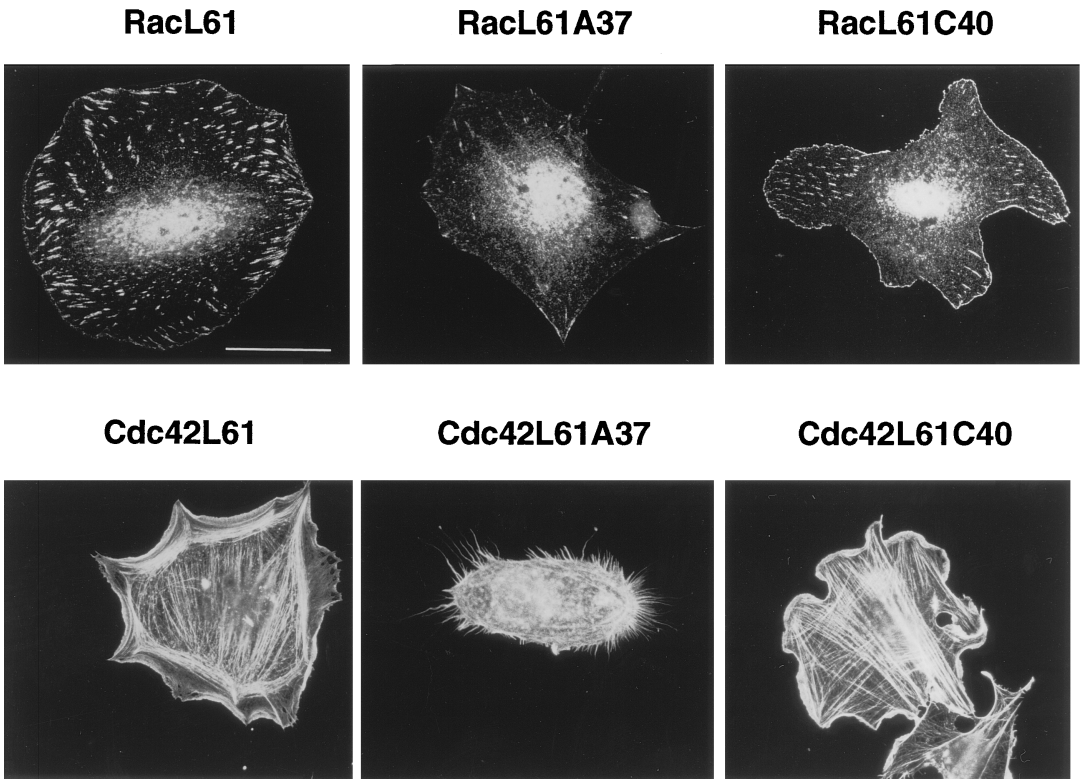

Figure 5. Cross-Talk between Cdc42, Rac, and Rho

To observe Rac-activation of Rho, serum-starved subconfluent Swiss 3T3 cells were fixed 4 hr after injection of the indicated small GTPase expression vectors (0.2 mg/ml) and focal adhesions were visualized with an anti-vinculin antibody and indirect immunofluorescence (top panel). To observe Cdc42-activation of Rac (and Rho), serum-starved subconfluent Swiss 3T3 cells were fixed 2 hr after injection of the indicated Cdc42 expression vectors (0.2 mg/ml) and F-Actin was visualized with fluorescent-tagged phalloidin (bottom panel). Injected cells were localized as described in Figure 3.

Cdc42 are still able to induce DNA synthesis, but that the F37A substitution in Rac completely blocks induction of DNA synthesis. We conclude that activation of $p65^{PAK}$ and the JNK/SAPK MAP kinase pathway are not required for Rac or Cdc42-induced G1 progression.

Discussion

The three related GTPases, Rho, Rac and Cdc42 play a variety of key roles in controlling cell behavior. Activation of Rho in mammalian cells leads to the assembly of actin stress fibers and associated focal adhesion complexes (Ridley and Hall 1992; Hotchin and Hall 1995), Rho is essential for G1 progression of the cell cycle (Yamamoto et al., 1993; Olson et al., 1995), and it is also required later for assembly of the contractile ring during cytokinesis (Mabuchi et al., 1993). Recently a number of candidate target proteins that interact with Rho in vitro in a GTP-dependent manner have been described; these include two ser/thr kinases, Protein Kinase N (PKN) and $p160^{ROCK}$, and three structural proteins rhophilin, rhotekin and citron (Ishizaki et al., 1996; Matsui et al., 1996: Leung et al., 1995; Amano et al., 1996; Watanabe et al., 1996; Madaule et al., 1995; Reid et al., 1996). A phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase) activity may also be controlled by Rho (leading to PIP_2 production), and although it is not yet clear that this is a direct interaction, it is of particular interest since PIP_2 is thought to be a key player in triggering actin polymerization and may also play a role in integrin complex assembly (Chong et al., 1994; Gillmore and Burridge, 1996).

Rac induces a dense and dynamic meshwork of actin filaments at the cell periphery to produce lamellipodia, whereas Cdc42 activation triggers filopodia extensions (Ridley et al., 1992; Kozma et al., 1995; Nobes and Hall, 1995). Both GTPases induce the assembly of structurally similar integrin complexes at the cell periphery (Nobes and Hall, 1995). Rac and Cdc42 also appear to fulfill an essential role during cell cycle progression; activated versions induce DNA synthesis when injected into quiescent Swiss 3T3 cells and dominant negative versions inhibit serum-induced growth (Olson et al., 1995). Furthermore, recent work has revealed that activation of Rac is an essential component of Ras-induced malignant transformation and that it may also play a role in the development of metastasis (Joneson et al., 1996; Michiels et al., 1995). The biochemical pathways involved in cell cycle control and, for Rac, in malignant transformation are unknown. Moreover, it is not clear

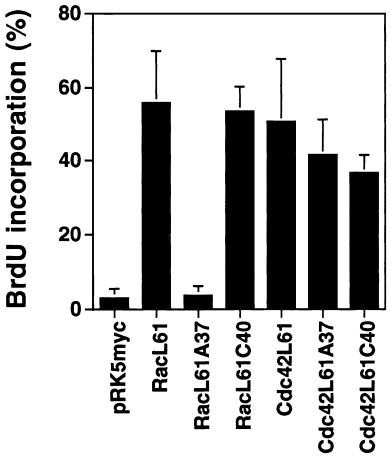

Figure 6. Stimulation of DNA Synthesis by Rac and Cdc42

Quiescent Swiss 3T3 cells were microinjected with pRK5myc (0.1 mg/ml), the indicated RacL61 expression vectors (0.1 mg/ml) or the indicated Cdc42L61 expression vectors (0.05 mg/ml) along with rat IgG (0.5 mg/ml) to localize injected cells. BrdU incorporation was monitored 40 hr later by immunofluorescence. Values are the percentage of injected cells positive for BrdU staining and correspond to the averages of at least three independent experiments in which 50–80 injected cells were scored per assay. Error bars correspond to the standard deviation.

whether they are the same or distinct from those that control actin polymerization and integrin complex assembly. A possible breakthrough in this analysis has been the discovery that both Rac and Cdc42 (but not Rho) can activate the JNK/SAPK and the p38/HOG MAP kinase cascades, thereby affecting gene transcription (Coso et al., 1995; Minden et al., 1995; Olson et al., 1995; Zhang et al., 1995; Bagrodia et al., 1995). However, these two MAP kinase pathways are usually associated with a stress response, and it is not clear whether they also contribute to cell proliferation and cell transformation.

In order to delineate the biochemical mechanisms underlying the biological effects of Rac and Cdc42, many groups have sought to identify cellular targets. At least five mammalian proteins have been identified that contain a CRIB motif: a tyrosine kinase $p120^{ACK}$; a ser/thr kinase $p65^{PAK}$; a member of the mixed lineage kinase family, MLK3; and two structural proteins, WASP (the product of the human Wisckott-Aldrich syndrome gene) and MSE55 (Manser et al., 1993, 1994; Burbelo et al., 1995; Aspenström et al., 1996). In addition, Rac has a highly specialized function in phagocytic cells where it regulates NADPH oxidase activity by interacting with a cytoplasmic target protein, $p67_{PHOX}$, which does not have a CRIB motif. In a yeast two-hybrid screen to identify Rho targets, we have isolated a partial clone of the ser/thr kinase, $p160^{ROCK}$. Although this and a second isoform, named rho-kinase or ROK, have been reported by others as Rho targets (Leung et al., 1995; Ishizaki et al., 1996; Matsui et al., 1996), we show here that in vitro at least, $p160^{ROCK}$ interacts with Rac in a GTP-dependent manner, almost as well as with Rho. A very modest stimulation of $p160^{ROCK}$ kinase activity by Rho has been reported both in vitro and in vivo, and it is not yet clear whether Rho or Rac can directly activate this enzyme or whether they act indirectly to localize it or to promote complex formation (Ishizaki et al., 1996).

In this paper we have addressed which of the target proteins are responsible for the following biological activities of Rac and Cdc42: (i) actin polymerization, (ii) integrin complex assembly, (iii) induction of DNA synthesis, (iv) cross-talk to downstream GTPase, and (v) activation of JNK/SAPK MAP kinase cascade. To do this we have introduced two selected amino acid substitutions (Y40C and F37A) into the effector loop of the two GTPases. These two substitutions were first shown to be highly selective in dissecting signaling pathways downstream of Ras, and we now report that they can be similarly used to look at Rac and Cdc42 function (White et al., 1995; Joneson et al., 1996). The Y40C mutation prevents the interaction of both Rac and Cdc42 with all CRIB-motif-containing proteins tested, including $p65^{PAK}$, WASP, MSE55, and the C. elegans protein, F09F7.5. This is further reflected by their inability to stimulate $p65^{PAK}$ kinase activity when the mutant GTPases and the wild-type kinase are cotransfected into COS cells. The Y40C mutation does not, however, interfere with the interaction of Rac with $p67_{PHOX}$ or with $p160^{ROCK}$, showing that its effects are selective (see Table 1). We show here that both RacL61C40 and Cdc42L61C40 are unable to activate the JNK/SAPK MAP kinase pathways; a result that is consistent with previous reports suggesting that activation of the MAP kinase cascades is mediated by stimulation of $p65^{PAK}$ (Bagrodia et al., 1995; Zhang et al., 1995; Brown et al., 1996).

Despite the loss of interaction with CRIB motif proteins, injection of Swiss 3T3 cells with RacL61C40 and Cdc42L61C40 leads to the induction of lamellipodia and filopodia, respectively, that is indistinguishable from that induced by nonmutated proteins. Furthermore, actin filament formation is accompanied by the assembly of vinculin-containing focal complexes at the cell periphery. We conclude from these experiments that the interaction of Rac and Cdc42 with CRIB motif proteins, and in particular $p65^{PAK}$, and the activation of MAP kinase pathways are not required for the induction of actin polymerization or integrin complex assembly. In agreement with this, we have found that the constitutively activated $p65^{PAK}$ does not induce any actin filament assembly when injected into serum-starved cells (data not shown). Recently, it has been reported that high level expression of WASP in endothelial cells induces condensation of filamentous actin in a Cdc42-dependent manner, leading to the suggestion that WASP links Cdc42 to the actin cytoskeleton (Symons et al., 1996). Since our experiments were done in fibroblasts and WASP is expressed only in hematopoietic cells, we cannot address this question, though we would speculate that if a fibroblast isoform of WASP exists, the consequences of its interacting with Cdc42 on the actin cytoskeleton must be subtle and not discernable in our microinjection experiments. Since the Y40C mutation in RacL61 does not interfere with $p67_{PHOX}$ or $p160^{ROCK}$ binding, these experiments do not rule out these or related proteins in lamellipodia formation. We have found, however, that a Y40K mutation in Rac inhibits its interaction with both $p67_{PHOX}$ and CRIB motif proteins, but not $p160^{ROCK}$. RacL61K40 also induces lamellipodia

Table 1. Summary of the RacL61 and Cdc42L61 Mutant Effects

Effectors	$p65^{PAK}$	$p160^{ROCK}$	$p67_{PHOX}$	$p65^{PAK}$ Activation	JNK Activation	Membrane Ruffling	Focal Complexes	Activation of rho	DNA Synthesis
RacL61	+	+	+	+	+	+	+	+	+
RacL61A37	+	−	+	+	+	−	−	−	−
RacL61C40	−	+	+	−	−	+	+	+	+
RacL61K40	−	+	−	−	−	+	+	+	+

Effectors	p50rhoGAP	$p65^{PAK}$	WASP	$p65^{PAK}$ Activation	JNK Activation	Filopodia	Focal Complexes	Activation of rac	DNA Synthesis
Cdc42L61	+	+	+	+	+	+	+	+	+
Cdc42L61A37	+	+	+	+	+	+	+	−	+
Cdc42L61C40	+	−	−	−	−	+	+	+	+

(+), active; (−), inactive

and focal complexes (data not shown) and it is unlikely that a $p67_{PHOX}$-related protein is involved.

RacL61A37 and Cdc42L61A37 interact normally with $p65^{PAK}$ in the in vitro binding assay and are able to activate $p65^{PAK}$ kinase activity to at least the same extent as the nonmutated versions in COS cell transfection assays. Despite this, stimulation of JNK1 activity by both F37A mutants is significantly reduced, raising the possibility that $p65^{PAK}$ might not be the only mediator of MAP kinase activation in these cells. Interestingly, MLK3, which interacts with Cdc42 and Rac in vitro, appears, from sequence analysis, to be related to the MAP kinase kinase kinase family of proteins (Holzman et al., 1994; Burbelo et al., 1995).

Microinjection of RacL61A37 into serum-starved subconfluent Swiss cells does not lead to the formation of lamellipodia or integrin complexes. It appears, therefore, that this mutation destroys Rac's interaction with an essential component of the downstream signaling pathway required for actin polymerization. Since this mutation blocks $p160^{ROCK}$ interaction, we speculate that this kinase may provide an essential signal required for Rac-induced cytoskeletal changes. Further analysis of $p160^{ROCK}$ will be required to address this question, and an approach we are currently undertaking is to identify mutations in $p160^{ROCK}$ that rescue the interaction with RacL61A37. It has also been suggested that Rac may control a PIP 5-kinase activity; whether this is affected by the F37A mutation will have to await further characterization of the lipid kinase and the nature of its interaction with the GTPase (Hartwig et al., 1995; Tolias et al., 1995). Somewhat surprisingly, the F37A mutation did not inhibit the ability of Cdc42 to induce filopodia or integrin complexes. Since we have ruled out CRIB motif–containing proteins as being required, we are left with no candidate Cdc42 targets that might account for filopodia formation. The F37A mutation in both Rac and Cdc42 blocks the cross-talk observed between GTPases in Swiss 3T3 cells, such that RacL61A37 is not able to activate Rho and Cdc42L61A37 no longer activates Rac. The latter mutation leads to an interesting effect; since this mutation does not interfere with filopodia induction, Cdc42L61A37 induces filopodia that are unaccompanied by subsequent Rac-mediated lamellipodia.

To analyze the effects of these mutations on cell cycle regulation, mutants were injected into quiescent Swiss 3T3 cells and their ability to induce DNA synthesis monitored. Both RacL61C40 and Cdc42L61C40 induce DNA synthesis almost as well as the nonmutated GTPases. We conclude that activation of $p65^{PAK}$ and the JNK/SAPK MAP kinase pathways are not required for induction of G1 progression and entry into S phase of the cell cycle. Cdc42L61A37 also induced DNA synthesis suggesting that this biological effect is direct and not dependent upon any cross-talk between Cdc42 and Rac. Rac L61A37, on the other hand, was unable to induce G1 progression in these cells despite being almost fully competent to activate $p65^{PAK}$ and the JNK/SAPK MAP kinase pathway. However, this substitution did inhibit actin polymerization and integrin complex assembly and, therefore, suggests the distinct possibility that the signal initiating G1 progression from Rac is generated as a consequence of integrin complex assembly or actin polymerization. Further mutational analysis may answer this question.

In conclusion, mutational analysis has revealed that the ability of Rac and Cdc42 to activate MAP kinase cascades and to induce cytoskeletal changes are mediated by distinct, independent downstream signals. Activation of MAP kinase pathways correlates well with activation of the $p65^{PAK}$ kinase, although a number of other CRIB motif–containing proteins interact with Rac and Cdc42 in a similar way and their biological roles are unclear. Activation of G1 progression by Rac and Cdc42 is also independent of MAP kinase activation and interaction with CRIB proteins. In the case of Rac, the ability to induce cell cycle progression correlates with actin polymerization and integrin complex asembly. It is possible, therefore, that these responses are interdependent. Rac has been shown to be one of several downstream targets of oncogenic Ras that are required for malignant transformation. Rac can also act as a weak oncogene itself and promote metastatic behavior. It will be of great interest to see which of the downstream signals controlled by Rac are involved in these pathways.

Experimental Procedures

Yeast Two-Hybrid System Screen

A cDNA encoding RhoL63 fused to the sequence encoding the GAL4 DNA-binding domain (GAL4DB) of the pYTH6 vector was stably integrated into the genome of the Saccharomyces cerevisiae strain Y190 (Aspenström and Olson, 1995). This fusion protein GAL4DB-RhoL63-expressing yeast strain was then transformed with a cDNA library from human HeLa cells (Clonetech) fused to the sequence

encoding the GAL4 activating domain in the pGAD GH vector. Transformants were screened for the activation of the reporter genes following the method described by Aspenström and Olson (1995). Of 45 positive clones recovered from the screen, 7 were still able to induce reporter gene expression when retransformed into Y190:pYTH6-RhoL63. One of those was shown on sequence analysis to code for aa 868–1030 of $p160^{ROCK}$ (Ishizaki et al., 1996).

Site-Directed Mutagenesis of RacL61 and Cdc42L61

Point mutations were introduced into pGEX-2T-RacL61 and into pGEX-2T-Cdc42L61 (Self and Hall, 1995) using the "Chameleon" double-stranded, site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. All mutated RacL61 and Cdc42L61 constructs were fully sequenced and the cDNAs subcloned as a BamHI–EcoRI fragment into the eukaryotic expression vector, pRK5, which had previously been modified so as to contain a myc epitope tag 5′ of the BamHI cloning site. DNA for microinjections and transfections was purified by CsCl gradient.

Expression and Purification of Recombinant Proteins

RacL61 (Rac1 isotype), Cdc42L61 (G25K isotype) and the various mutants were expressed in E. coli as glutathione S-transferase (GST) fusion proteins and purified on glutathione–sepharose beads as described earlier (Self and Hall, 1995). The recombinant GTPases were released from the beads by cleavage with human thrombin (Sigma, 5 U for 1L bacterial culture) and thrombin removed by adding 10 μl of p-aminobenzamidine-agarose beads (Sigma) for 30 min at 4°C. Purified proteins were dialyzed against 15 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 0.1 mM dithiothreitol (DTT) and concentrated by ultrafiltration using Centricon-10 (Amicon). Active protein concentrations were determined by the filter binding assay using [^{3}H]-GTP as previously described (Ridley et al., 1992). The catalytic C-terminal domain (residues 198–439) of p50rhoGAP (Lancaster et al., 1994), full-length murine $p65^{PAK}$ (Burbelo et al., 1995), aa 201–321 of human WASP (Aspenström et al., 1996), aa 1–199 of $p67_{PHOX}$ (Diekmann et al., 1994), and human $p160^{ROCK}$ (residues 868–1030) were all expressed as GST fusion proteins in E. coli. Fusion proteins were eluted from the beads with 5 mM reduced glutathione (Sigma) and concentrated by ultrafiltration using centricon-10. Protein concentration was assayed following the method of Bradford and purity of protein preparations was visualized on Coomassie blue–stained SDS–polyacrylamide gels.

Dot Blot Assay

The interaction of putative target proteins with Rac, Cdc42, and the various mutants was assessed using a dot blot assay as previously described (Diekmann et al., 1995). In brief, 10 μg of GST fusion protein, p50rhoGAP, $p67_{PHOX}$, $p160^{ROCK}$, $p65^{PAK}$, or WASP were spotted in a volume of 5 μl onto nitrocellulose membranes, the filter was air dried and incubated with blocking buffer (1 M glycine, 5% milk powder, 1% ovalbumin, and 5% fetal calf serum) for 2 hr at room temperature. The membrane was washed in buffer A (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 5 mM $MgCl_2$, and 0.1 mM DTT) and incubated for 5 min at 4°C with [γ-^{32}P]GTP-bound proteins in buffer A. The filters were quickly washed three times with cold buffer A containing 0.1% tween and interacting GTPases visualized by autoradiography. Quantitative measurements of bound GTPase were carried out by cutting the individual spots and Cerenkov counting.

Cell Culture and Microinjection

Swiss 3T3 cells and Cos-1 cells are cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics. Preparation of confluent, serum-starved Swiss 3T3 cells: cells were plated in serum at a density of 6×10^4 onto acid-washed coverslips that had been etched with a cross to aid subsequent localization of injected cells. Seven to 10 days after seeding, the cells become quiescent at which time they were serum-starved for 16 hr in DMEM containing 2 g/l $NaHCO_3$. Preparation of subconfluent, serum-starved Swiss 3T3 cells; cells were plated at a density of 1×10^5 into 60 mm dishes. After 7 to 10 days, cells were serum-starved for 16 hr, trypsinized for 1 min, and resuspended in serum-free DMEM containing 0.5 mg/ml soybean trypsin inhibitor (Sigma). The cells were pelleted and resuspended in serum-free medium before being replated at a density of 6×10^4 onto coverslips treated overnight at 4°C with fibronectin (50 μg/ml). Cells were allowed to attach and spread for 1 hr before microinjection. Eukaryotic expression vectors (pRK5-myc) encoding the various rac and cdc42 mutants were microinjected alone or with either Texas red–coupled dextran (5 mg/ml) or rat immunoglobulin (0.5 mg/ml) to mark injected cells, into the nucleus of ~50 cells over a period of 10 min. Cells were returned to an incubator for a further 2 hr or 4 hr as indicated before fixation. During microinjection, cells were maintained at 37°C with an atmosphere of 10% CO_2.

Immunofluorescence Microscopy

At the indicated times, microinjected Swiss 3T3 cells were rinsed in PBS and fixed for 10 min in freshly prepared 4% (w/v) paraformaldehyde. All steps were carried out at room temperature, and coverslips were rinsed in PBS between each of the steps. Cells were permeabilized in a solution of 0.2% Triton X-100 for 5 min, and free aldehyde groups were reduced with 0.5 mg/ml sodium borohydride for 10 min. Cells were doubled-labeled following the procedure previously described (Nobes and Hall, 1995). In brief, cells were incubated in the presence of the primary monoclonal antibodies anti-myc or anti-vinculin (Sigma) diluted in PBS, for 60 min. Coverslips were transferred to a second antibody mixture composed of FITC-conjugated goat anti-mouse antibody (Sigma) and TRITC-conjugated phalloidin (Sigma) for 30 min. For vinculin staining, cells were incubated in the presence of a tertiary FITC-conjugated donkey anti-goat antibody for an additional 30 min. Coverslips were mounted by inverting them onto 10 μl moviol mountant containing p-phenylenediamine (1 mg/ml) as an antibleach agent. After 2 hr at room temperature, the coverslips were examined on a Zeiss axiophot microscope using Zeiss 40×1.3 and 63×1.4 oil immersion objectives. Fluorescence images were recorded on Kodak T-MAX 400ASA film.

DNA Synthesis

Swiss 3T3 cells were plated in serum at a density of 5×10^4 cells per coverslip. Seven to 9 days after seeding, confluent, quiescent cells were microinjected in serum-free medium over a period of 10 min before being returned to a 37°C incubator. Cells were incubated with 10 μg/ml BrdU for 40 hr before fixation in 4% (w/v) paraformaldehyde in PBS. Cells were stained for rat IgG to localize injected cells, and BrdU incorporation was monitored using an anti-BrdU antibody as reported earlier (Olson et al., 1995).

Mammalian Cell Transfections

Cos-1 cells were transfected by the DEAE-dextran method as described (Olson et al., 1995). Plasmid amounts per 10 cm petri dish were as follows: 5 μg of pRK5myc-$p65^{PAK}$L107F, pRK5myc-$p65^{PAK}$ or 5 μg of pCMV-FLAG-JNK1 with or without 1.5 μg each of pRK5myc, pRK5myc-RacL61, pRK5myc-Cdc42L61, or the various Rac and Cdc42 mutants. Twenty-four hours later, transfected cells were serum-starved for 16 hr prior to lysis in the respective JNK1 or $p65^{PAK}$ buffers.

$p65^{PAK}$ Kinase Assay

Cos-1 transfected cells were lysed in 50 mM Tris–HCl [pH 8]; 150 NaCl; 1mM EDTA; 0.1% Triton X-100; 50 mM NaF; 0.1 mM vanadate, and protease inhibitors (buffer B). To quantitate the amount of $p65^{PAK}$ present in each experiment, one-tenth of each lysate was loaded onto 15% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. Myc-epitope-tagged proteins were visualized with the anti-myc monoclonal antibody and 0.1 μCi/ml of protein A-^{125}I (Amersham) and quantitated by phosphorimage analysis. An equal amount of $p65^{PAK}$ was immunoprecipitated from each lysate with the anti-myc antibody in buffer B for 1 hr at 4°C. Twenty-five microliters of protein G-sepharose beads 50% (v/v) (Sigma) were added and the slurry incubated at 4°C for an additional hour. The immune complex was washed three times in buffer B and once in buffer C (20 mM Tris–HCl [pH 7.5]; 20 mM $MgCl_2$; 10 mM $MnCl_2$; 1 mM EDTA; 1 mM EGTA; 40 μM ATP). $p65^{PAK}$ kinase activity was assayed for 10 min at 30°C in buffer C containing 5 μg of MBP and 5 μCi of [γ-^{32}P]-ATP. The reaction was stopped

by adding electrophoresis sample buffer and proteins were separated on 15% SDS–PAGE, transferred to nitrocellulose, and visualized by autoradiography. The relative levels of MBP phosphorylation were determined by phosphorimage analysis. Levels of $p65^{PAK}$ immunoprecipitated were checked by Western blotting with anti-myc antibodies and proteine A-^{125}I (Amersham).

JNK1 Kinase Assay

Transfected Cos-1 cells were lysed and harvested in 25 mM HEPES (pH 7.6); 1% (v/v) Triton X-100; 1% (w/v) sodium deoxycholate; 0.1% (w/v) SDS, 0.3 M NaCl; 50 mM NaF; 0.1 mM vanadate; 5 mM EDTA; 5 mM EGTA; 40 mM sodium pyrophosphate and protease inhibitors. JNK1 kinase activity in cell extracts was measured in immunocomplexes using GST-c-jun as a substrate and as described previously (Olson et al., 1995). The relative levels of c-jun phosphorylation were determined by phosphorimager after gel electrophoresis. The amount of immunoprecipitated JNK1 was evaluated on Western blots using anti-FLAG (IBI) antibody and revealed by chemiluminescence.

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