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# Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton

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The actin cytoskeleton plays a fundamental role in all eukaryotic cells—it is a major determinant of cell morphology and polarity and the assembly and disassembly of filamentous actin structures provides a driving force for dynamic processes such as cell motility, phagocytosis, growth cone guidance and cytokinesis. The ability to reorganize actin filaments is a fundamental property of embryonic cells during development; the shape changes accompanying gastrulation and dorsal closure, for example, are dependent on the plasticity of the actin cytoskeleton, while the ability of cells or cell extensions, such as axons, to migrate within the developing embryo requires rapid and spatially organized changes to the actin cytoskeleton in response to the external environment. Work in mammalian cells over the last decade has demonstrated the central role played by the highly conserved Rho family of small GTPases in signal transduction pathways that link plasma membrane receptors to the organization of the actin cytoskeleton.

**Keywords:** GTPase; actin; Rho; migration

## 1. INTRODUCTION

The mammalian Rho family of small GTPases currently comprises eight distinct members: Rho (A, B, C isoforms), Rac (1, 2, 3 isoforms), Cdc42 (G25K, Cdc42Hs isoforms), RhoD, RhoG, TC10, Rnd (Rnd1, RhoE/Rnd3, Rnd6) and TTF, which share around 50–55% identity to each other. The consequences of activating Rho, Rac and Cdc42 in Swiss 3T3 fibroblasts have been well characterized. Rho regulates the assembly of actin stress fibres (figure 1), Rac regulates the polymerization of actin at the cell periphery to produce lamellipodia and membrane ruffles, while Cdc42 triggers filopodia formation (Ridley & Hall 1992; Ridley *et al*. 1992; Nobes & Hall 1995; Kozma *et al*. 1995; Puls *et al*. 1999). In addition, all three GTPases regulate the formation of cell–matrix adhesion sites called focal adhesions, which are intimately associated with the actin structures (Nobes & Hall 1995; Hotchin & Hall 1995). Furthermore, the GTPases appear to be linked to one another in a cascade; activation of Cdc42, for example, leads to rapid localized activation of Rac (Nobes & Hall 1995). It has been suggested, therefore, that these GTPases may be important regulators of cell movement in response to extracellular signals (Hall 1998; Lauffenburger & Horwitz 1996).

In addition to regulating the organization of the actin cytoskeleton, Rho-related GTPases regulate gene transcription. Rac and Cdc42 have been shown, in a variety of cell types, to activate the JNK and p38 MAP kinase pathways, while Rho has been shown to activate the serum response factor, through an unknown signal transduction pathway (Coso *et al*. 1995; Minden *et al*. 1995; Hill *et al*. 1995). This dual role of Rho/Rac/Cdc42 in regulating actin organization and gene transcription appears to be conserved in yeast and *Drosophila* (Leberer *et al*. 1997; Glise & Noselli 1997). A number of other cellular activities have been ascribed to these GTPases; Rho and Rac activity are required for the assembly of cadherin-based, cell–cell junctions in human epithelial cells, while Rac regulates the NADPH oxidase enzyme complex in phagocytic cells (Braga *et al*. 1997; Abo *et al*. 1991; Diekmann *et al*. 1994). It appears, therefore, that Rho GTPases regulate the organization of the actin cytoskeleton along with other cellular processes to promote coordinated changes in cell behaviour (Hall 1998).

## 2. RHO GTPASE SIGNALLING PATHWAYS

Signalling through Rho GTPases can be initiated by activation of many different types of plasma membrane receptor, including tyrosine kinase, G-protein-coupled and cytokine receptors. It is believed that receptors are able to activate guanine nucleotide exchange factors (GEFs) that catalyse loss of GDP and allow binding of GTP, but the biochemical mechanisms involved are still poorly understood. More than 30 mammalian Rho GEFs have been identified to date and all are characterized by the presence of a Dbl homology domain, which catalyses the exchange reaction, followed immediately by a pleckstrin homology domain. GEFs often contain other protein–protein interaction motifs that vary between different family members (Cerione & Zheng 1996).

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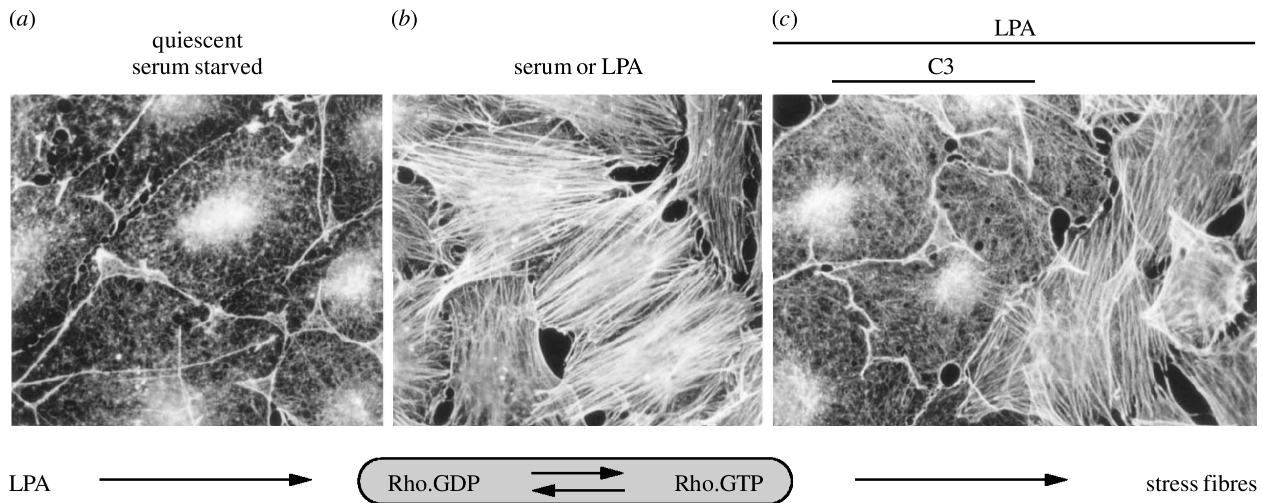


Figure 1. Rho regulates the assembly of actin stress fibres induced by lysophosphatidic acid (LPA). (*a*) Quiescent, serum-starved Swiss 3T3 fibroblasts have very little organized actin cytoskeleton as visualized by rhodamine phalloidin, but treatment with serum or LPA (*b*) induces actin:myosin stress fibres within 15 min. If cells are microinjected with the Rho inhibitor, C3 transferase, before LPA addition (*c*), stress fibre assembly is inhibited. This suggests that the Rho GTPase regulates a signal transduction pathway in these fibroblasts linking the LPA receptor to the assembly of actin stress fibres (Ridley & Hall 1992). Using similar approaches (data not shown), it was established that platelet-derived growth factor or insulin activates the Rac GTPase in quiescent Swiss cells and that this leads to the assembly of actin filaments at the cell periphery to form lamellipodia and membrane ruffles (Ridley *et al*. 1992). Bradykinin, tumour necrosis factor-α and interleukin-1 activate Cdc42 in these cells leading to actin filament assembly at the periphery to produce filopodia (Nobes & Hall 1995; Kozma *et al*. 1995; Puls *et al*. 1999).

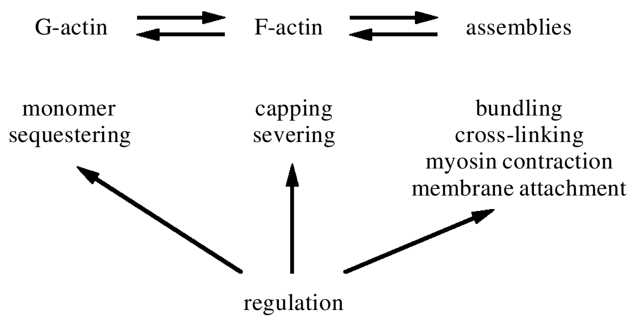


Figure 2. Actin filament assembly. Monomeric (G-)actin is sequestered in cells by monomer-sequestering proteins such as thymosin and profilin. New actin filaments can be induced *de novo* by nucleation of actin monomers or pre-exisiting filaments can be extended by uncapping of filament ends or by creating new ends by severing. These activities appear to occur at membranes and may be facilitated by lipids such as phosphatidylinositol-4,5-bisphosphate. Actin filaments can be organized into higher-order structures by bundling or cross-linking proteins. Cross-linking induced by myosin II, leads to the formation of contractile filaments that can affect cell shape. It is possible that Rho GTPases can regulate one, or more likely several, of these steps.

Exchange assays done *in vitro* and *in vivo* suggest that some GEFs can potentially act on multiple GTPases, e.g. Vav on Cdc42, Rac and Rho (Olson *et al*. 1996), while others are more specific, e.g. lbc and p115RhoGEF act on Rho (Hart *et al*. 1996), Tiam-1 acts on Rac (Michiels *et al*. 1995) and FGD1 acts on Cdc42 (Olson *et al*. 1996).

Once activated, Rho GTPases mediate their cellular effects through interaction with target proteins and one of the surprises of the last few years has been the large number (over 20) of targets identified for Rho, Rac and Cdc42. Targets are defined as proteins that interact directly with the GTP-bound, but not the GDP-bound, form of a GTPase and so far these include tyrosine kinases, serine or threonine kinases, phospholipases, lipid kinases and many scaffold-like or structural proteins. While there are data linking some of these molecules to changes in the actin cytoskeleton (see figure 2), the roles of many are unknown. There are many excellent reviews on this topic (e.g. Van Aelst & D'Souza-Schorey 1997).

## 3. CELLULAR RESPONSES CONTROLLED BY RHO GTPASES

Genetic analysis in yeast has revealed the complexity of activities associated with these proteins and while the biochemical details of their function in yeast and mammalian cells may have diverged, some of the general lessons learnt in yeast (see figure 3) are likely to be useful in thinking about Rho GTPase function in higher eukaryotes. The first is that a single GTPase can regulate multiple pathways to promote a coordinated biological response. In this case Rho1p in yeast has been shown to control the activity of at least three proteins each of which is required for bud growth. The large number of target proteins and functional activities reported for mammalian Rho GTPases suggests that this is also likely to be true in higher eukaryotes, but to date there are few, if any, examples in a mammalian cell where a single Rho GTPase has been shown to control two or more activities that together contribute to a particular biological response. The second lesson from yeast is that members of the Rho family can cooperate with each other to promote a response. In the example shown, Cdc42 is required to establish a polarized bud and Rho then acts to cause growth of the bud. Finally, Ras and Rho GTPases

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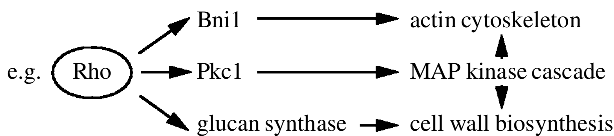
(i) Rho GTPases regulate multiple signalling pathways:




(ii) Rho GTPases cooperate with each other:

e.g. (bud establishment) Cdc42 + Rho (bud maintenance)

(iii) Ras and Rho GTPases cooperate with each other:

e.g. (bud site selection) Bud1 + Cdc42 (bud establishment)

Figure 3. General features of Rho GTPases in yeast. Genetic analysis of Rho1 and Cdc42 in yeast has led to a reasonably clear picture of at least some of their biological roles. Three general lessons have emerged that have shaped some of our thinking about the possible roles of these proteins in higher eukaryotes: (i) Rho GTPases can regulate multiple pathways to promote a coordinated biological response. In the example shown, Rho1, which is required for bud growth, regulates three pathways, a pkc1-dependent MAP kinase pathway affecting gene transcription, an enzyme required for cell wall synthesis and a structural protein required for the correct organization of the actin cytoskeleton. (ii) Different Rho GTPases can cooperate with each other to produce a coordinated response. In the example shown, Cdc42 is required for initiation of bud assembly at the correct site, while Rho1 is then required for continued growth of the bud. (iii) Rho GTPases can cooperate with Ras GTPases. In the example shown, Bud1, a Ras-like GTPase, is required for the selection of the bud site on the yeast surface. This then defines the sites at which Cdc42 subsequently acts.

cooperate with each other and again in the example of bud formation, Budl (a Ras-like GTPase) is required for bud site selection, and this then defines the site at which Cdc42 acts. One of the aims of our laboratories is to explore these possibilities in the context of a mammalian cell.

Since Rho GTPases clearly play an important role in regulating actin assembly and disassembly, we have concentrated our efforts on processes where actin is known to play an important role. Table 1 lists some of the important roles of the actin cytoskeleton in eukaryotic cells. We have so far concentrated our efforts on events that are dependent on dynamic changes to the actin cytoskeleton and in particular cell movement, axon guidance and phagocytosis. Here we will describe our results so far on the role of Ras and Rho GTPases in cell movement.

## 4. THE ROLES OF RAS AND RHO GTPASES IN CELL MIGRATION

### (a) *The migration assay*

To analyse the role of Rho GTPases in a simple cell migration assay, we have introduced scratch wounds (made with a microinjection needle) into confluent monolayers of primary rat embryo fibroblasts (REFs). The wounds are around three to four cell diameters in width

Table 1. *Some aspects of cell behaviour that are dependent on the actin cytoskeleton*

| | behaviour |
|---|---|
| structural | cortical cytoskeleton<br>cell morphology<br>polarity<br>adhesion complex<br>vesicle trafficking |
| dynamic | cell movement<br>axon guidance<br>phagocytosis<br>cytokinesis<br>bacterial invasion |

and closure of control cells takes 5–6 h in serum. Close inspection of wound edge cells using time-lapse video microscopy reveals that cells fill the space by migration and not simply by spreading, and that on average, a cell at the wound edge moves a distance of around 1.5–2 cell diameters. Addition of cycloheximide has no effect on migration in these short-term assays, suggesting that no new gene transcription is required. Finally, it is clear from the video recordings (figure 4*a*) that cells move coordinately as a sheet and not as individuals. Cells at the wound margin have a highly dynamic leading edge showing lamellipodia, filopodia and membrane ruffles, while cell borders in contact with neighbouring cells are quiescent and show little dynamic activity. The cells have, therefore, a polarized morphology during migration.

To analyse the role of GTPases, we have microinjected cells (a patch of around 50 on either side of a wound) with inhibitors of (i) Rho (C3 transferase), (ii) Rac (dominant negative, N17Rac protein prepared in *Escherichia coli*), (iii) Cdc42 (dominant negative, N17Cdc42 protein expressed in *E. coli*, or a Cdc42-binding fragment derived from WASP), or (iv) Ras (a neutralizing monoclonal antibody, Y13–259).

### (b) *The role of Rac*

As shown in figure 4*d*, microinjection of fluorescent dextran, as a control, has no effect on the migratory properties of the cells. However, figure 4*b* shows that inhibition of Rac completely prevents cell movement. Closer inspection of the cells reveals that the dynamic activity of the leading edge seen in control cells is completely suppressed, though a few filopodia may still remain. We conclude that Rac, presumably through its ability to promote actin polymerization at the cell periphery, provides the driving force for the protrusive activity required for cell migration.

### (c) *The role of Cdc42*

Inhibition of Cdc42, using either of two distinct inhibitors (a dominant negative version of Cdc42 or a fragment derived from a Cdc42 target protein), inhibits cell migration by *ca*. 50%. Closer inspection of the cells reveals that they have lost their polarity. Instead of making protrusions only at the leading edge, cells are now seen to have protrusions all around the cell periphery and pushing underneath neighbouring cells.

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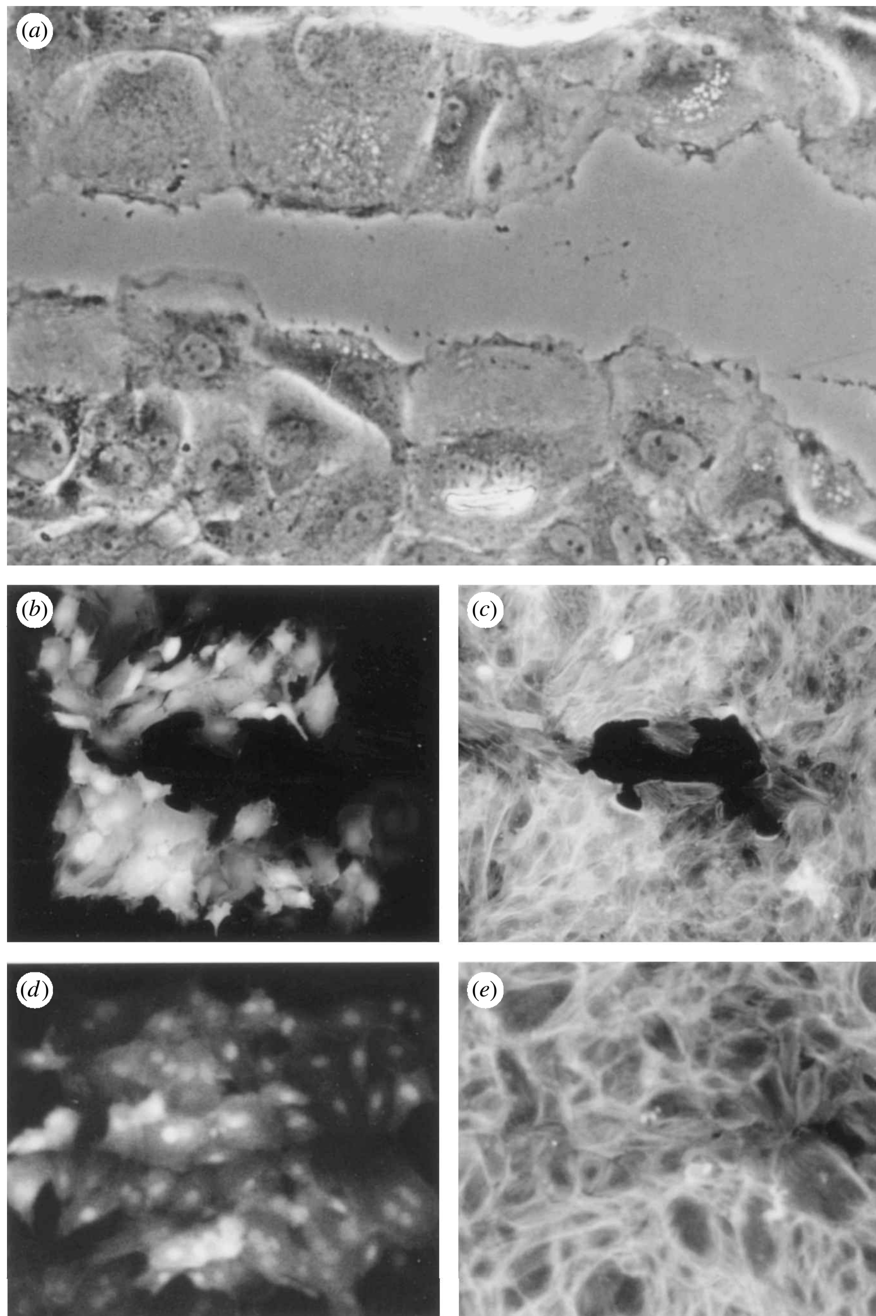


Figure 4. A wound-healing assay using primary REFs. To analyse the role of Ras and Rho GTPases, inhibitors were microinjected into a patch of *ca.* 50 cells on either side of a wounded monolayer. The time taken for subsequent wound closure is compared with that of control, marker-injected cells. (*a*) In phase contrast, polarized cells at the wound margin, with highly dynamic leading edges (lamellipodia, ruffles and filopodia) and quiescent borders where cells are in contact with their neighbours. (*d*, *e*) Cells around a wound were injected with fluorescent dextran (*d*) and then left for 6 h. It can be seen by phalloidin fluorescence that the injection procedure does not affect migration and wound closure (*e*). (*b*, *c*) Cells were injected with dominant negative Rac (*b*) and this completely inhibited the movement of the injected cells (visualized with phalloidin), seen after 6 h (*c*).

During cell migration, the Golgi, which normally occupies a position extending over about one-third of the nuclear perimeter, reorientates in the direction of migration. Reorientation of the Golgi to face the wound is a rather slow process increasing from 33% to *ca.* 70–80% over a period of around 6 h. It is unlikely, therefore, that Golgi reorientation is a driving force for movement, but it presumably facilitates migration by delivering new membranes to the leading edge (Schliwa *et al.* 1999). Inhibition of Cdc42 also prevents Golgi reorganization. We conclude that Cdc42 is required to define the polarity of the migrating cell and that this is reflected in the localization of Rac-dependent protrusive activity to the leading edge and in the reorganization of the Golgi, presumably in a microtubule-dependent manner, towards the leading edge.

### (d) *The role of Rho*

REFs have very prominent stress fibres and focal adhesion complexes and these are maintained during

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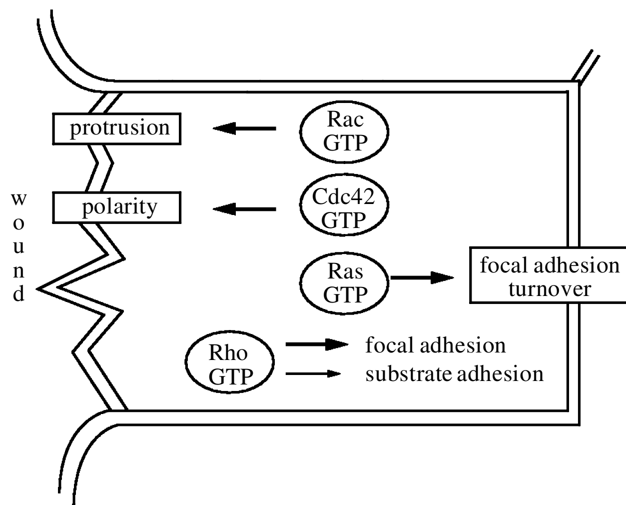


Figure 5. At least four small GTPases cooperate to promote cell migration during a fibroblast wound healing assay.

migration initiated by wounding of the monolayer. As recently described by Smilenov *et al.* (1999), cells appear to migrate through static focal adhesions and when these adhesive structures reach the rear of the migrating cell they dissipate. To determine whether focal adhesions nevertheless slow down cell migration, we have removed focal adhesions and stress fibres in the migrating cells by addition of the compound Y27632, an inhibitor of p160Rho kinase, an essential downstream target of Rho required for stress fibre assembly (Watanabe *et al.* 1999). Under these conditions, cells move *ca.* 50% faster than non-treated cells and we conclude that although cells can regulate turnover of focal adhesions during migration, cells move faster without them. It also shows that actin:-myosin filaments, at least in the form of stress fibres, are not required for migration.

We also attempted to repeat this experiment by directly inhibiting Rho by microinjection of cells with C3 transferase. We found that at concentrations of C3 just sufficient to remove all visible focal adhesions cells moved similar to control cells, confirming that stress fibres are not needed for migration. However, injection of C3 transferase at threefold higher concentrations caused significant rounding up and detachment of cells, and cell migration was inhibited. This experiment strongly suggests that Rho has other activities, distinct from stress fibre and focal adhesion formation, and related to adhesion and spreading.

### (e) *The role of Ras*

Several groups have previously described the importance of Ras in cell migration (Fox *et al.* 1994). Immunofluorescence analysis using an antibody specific to dually phosphorylated ERK (a marker for Ras activation), revealed that ERK and Ras are activated 5 min after wounding, though ERK fluorescence returns to background levels within an hour. Interestingly, phospho-ERK is detected up to eight cells back from the wound margin—we do not know the mechanism by which cells distant from the wound are activated.

To determine whether Ras is required for migration, cells were microinjected with a Ras-neutralizing monoclonal antibody. Under these conditions cell movement is almost completely blocked. The reasons for this were not entirely clear at first, since cells still have a polarized and dynamic leading edge. We did notice, however, some thickening of stress fibres and focal adhesions in injected cells and wondered if there might be a functional relationship between Ras and Rho. To test this idea, cells were microinjected with the neutralizing Ras antibody and at the same time treated with Y27632 to remove focal adhesions. Under these conditions cells moved at a similar speed to control cells. Our interpretation of this result is that Ras is required for focal adhesion turnover. Addition of a MEK inhibitor (PD98059) or a PI 3-kinase inhibitor (wortmannin) had only minor effects on cell migration.

## 5. CONCLUSIONS

Figure 5 summarizes our conclusions concerning the role of four small GTPases (Rho, Rac, Cdc42 and Ras) in the migration of primary embryo fibroblasts (Nobes & Hall 1999). Rac is required to induce the protrusive activity at the front of the cell that we believe is the driving force for movement. Cdc42 is required to maintain the polarity of the migrating cell; it ensures that Rac activity is restricted to the leading edge and that microtubules and Golgi reorientates in the direction of migration. This nicely demonstrates the cooperation between these two GTPases to promote directed migration. A similar conclusion has been reached by others in experiments analysing chemotaxis of macrophages in a gradient of CSF-1 (Allen *et al.* 1998). The molecular mechanisms by which Cdc42 controls cell polarity are not clear; perhaps it localizes Rac, or a critical Rac target, to the leading edge. Alternatively, it may inhibit Rac activity at sites of cell–cell contact.

Rho appears to be active both in confluent monolayers and in migrating cells, since cells have prominent stress fibres and focal adhesions. To move, however, matrix adhesions must be turned over and it appears that this occurs predominantly at the rear of the migrating cell. Our data suggest that in these cells at least, this is controlled by Ras. The molecular link between Ras and focal adhesions is unclear; it could be that Ras affects components of the focal adhesion itself, or that Ras affects the activity of Rho. One interesting possibility is that the Ras effect is mediated by p120RasGAP, which is known to form a complex with p190RhoGAP, a down-regulator of Rho.

Future work will focus on the molecular pathways that are regulated by Rho and Ras GTPases and that contribute to movement. We are also interested in analysing movement in other cell types to see whether cells can move in different ways and make different uses of GTPases. Growth cone guidance of axons can be thought of as a highly specialized form of cell migration (where the cell body does not translocate) and we are, therefore, also interested in exploring the roles of Rho family and other small GTPases in this process. Finally, a crucial component of directed migration is the spatial organization of these and other signalling molecules. We currently have no idea how this is determined and the early work on the activation of signalling pathways by addition of

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factors to cells in culture is unlikely to be able to address this question. Relatively simple assays, such as the one described here, will be essential if the spatial organization of GTPase-controlled signals are to be addressed.

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