Rho GTPases and cell migration

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Summary

Cell migration involves dynamic and spatially regulated changes to the cytoskeleton and cell adhesion. The Rho GTPases play key roles in coordinating the cellular responses required for cell migration. Recent research has revealed new molecular links between Rho family proteins and the actin cytoskeleton, showing that they act to regulate actin polymerization, depolymerization and the activity of actin-associated myosins. In addition, studies on integrin signalling suggest that the substratum continuously feeds signals to Rho proteins in migrating cells to influence migration rate. There is also increasing evidence that Rho proteins affect the organization of the microtubule and intermediate filament networks and that this is important for cell migration.

Key words: Rho GTPases, Actin, Cytoskeleton, Migration, Cell adhesion

Introduction

In multicellular organisms, cell migration is essential to normal development, and is required throughout life for responses to tissue damage and infection. Cell migration also occurs in chronic human diseases; in cancer, atherosclerosis and chronic inflammatory diseases such as rheumatoid arthritis, preventing the migration of specific cell types could significantly inhibit disease progression.

Cell migration is a multistep process involving changes in the cytoskeleton, cell-substrate adhesions and the extracellular matrix. Many cell types migrate as individual cells, including leukocytes, lymphocytes, fibroblasts and neuronal cells, but epithelial cells and endothelial cells often move as sheets or groups of cells - for example, in duct development, in healing a wound and in angiogenesis.

Cell migration is usually initiated in response to extracellular cues, which can be diffusible factors, signals on neighbouring cells, and/or signals from the extracellular matrix. These cues then stimulate transmembrane receptors to initiate intracellular signalling. Many different intracellular signalling molecules have been implicated in cell migration, including small GTPases, Ca^{2+}-regulated proteins, mitogen-activated protein kinase (MAPK) cascades, protein kinases C, phosphatidylinositide kinases, phospholipases C and D, and tyrosine kinases. The involvement of all these signalling molecules is not surprising given the diversity of extracellular signals that affect cell migration, and the number of cellular responses that have to be coordinated. The concept that Rho family GTPases could regulate cell migration stems from observations that they mediate the formation of specific actin-containing structures (Van Aelst and D'Souza-Schorey, 1997; Hall, 1998). Subsequently, Rho proteins have been found to regulate several other processes relevant to cell migration, including cell-substrate adhesion, cell-cell adhesion, protein secretion, vesicle trafficking and transcription. Here, I discuss recent insights into how Rho proteins and their signalling partners contribute to cell migration, focusing in particular on the cytoskeleton and cell-substrate adhesion.

Rho proteins: the tools

At least 20 Rho family proteins have so far been identified in humans (Table 1), and of these RhoA/B, Rac1/2 and Cdc42 have been the most widely studied for their effects on cell migration. Many other members can affect actin organization, however, and are therefore expected to influence cell migration. In *Drosophila melanogaster* there are seven Rho family members, and in *Caenorhabditis elegans* there are five (Table 1), most of which have been implicated in cell migration and/or morphogenesis. In *Dictyostelium discoideum*, there are at least 15 members of the Rho family (Rivero et al., 2001), of which seven can be loosely grouped into a Rac-like subfamily, one is part of a new RhoBTB family (which also includes one member in *Drosophila* and at least two members in humans), whereas the others are not closely related to any specific human Rho family member. There is also a plant-specific Rho subfamily, the Rop proteins (Winge et al., 2000).

Rho proteins generally cycle between an active, GTP-bound, conformation and an inactive GDP-bound conformation (Fig. 1). In the GTP-bound form, they interact with downstream target proteins to induce cellular responses (for a recent extensive review of Rho targets see Schmitz et al., 2000). Rho proteins can exchange nucleotide and hydrolyse GTP at slow rates in vitro, and these reactions are catalysed by guanine-nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs), respectively. In addition, Rho proteins can bind to proteins known as GDIs (guanine-nucleotide-dissociation inhibitors; Fig. 1), which prevent their interaction with the plasma membrane but not necessarily with downstream targets (Carpenter et al., 1999; Hansen and Nelson, 2001). Most Rho proteins are post-translationally modified at their C-termini by prenylation of a conserved cysteine (Fig. 1), and this is required for their interaction with membranes (Seabra, 1998). Two classes of point mutant have been used extensively to analyse Rho protein function: first, activated mutants, which are constitutively GTP-bound because the GTPase activity is inhibited (Fig. 1); second, dominant negative mutants, which generally have reduced

Table 1. Rho family members in humans, *Drosophila melanogaster*, *C. elegans* and *Dictyostelium discoideum*

Subfamily	Humans	*Drosophila*	*C. elegans*	*Dictyostelium*
Rho	RhoA, B, C	Rho1	Rho1	None
Rac	Rac1*,2, Rac3/1B	Rac1,2	Rac1/CED10, Rac2	Rac1a,b,c, RacF1,2, RacB
Cdc42h	Cdc42/G25K*, TC10 TCL, Chp1,2	Cdc42	Cdc42	None
Mig2	RhoG	Mt1/Mig2-like	Mig2	None
Rnd	Rnd1,2, RhoE/Rnd3	None	None	None
RhoBTB	RhoBTB1,2	RhoBTB	None	RacA
Others (not classified in subfamilies)	RhoD, Rif, TTF/RhoH	RhoL/Rac3	None	RacC*,D,E, RacG-J,L

*Splice variants known.

affinity for nucleotides (Self and Hall, 1995) and are likely to titrate out GEFs (Feig, 1999). It is interesting that dominant negative mutants appear to be selective in inhibiting the action of one Rho family member, at least when Rho, Rac and Cdc42 are compared, and this is likely to be because each protein has a different localization in cells. So far, however, it is not known whether dominant negative Cdc42 can inhibit, for example, the activation of a closely related protein such as TC10 (Table 1).

A number of bacterial toxins covalently modify some of the Rho family members and either activate (deamidation) or inactivate (ribosylation, glucosylation) them (Lerm et al., 2000). Although these toxins have been very useful for assessing Rho protein involvement in cellular responses, the majority of Rho family proteins have not been tested for their ability to be modified by each of these toxins, and so it is not possible to assign effects of a toxin to inhibition or activation of a particular family member. C3 transferase from *Clostridium botulinum* is an exception in that so far it appears to be specific for RhoA, B and C (Wilde et al., 2000).

Members of the Rnd subfamily of Rho proteins appear to have a mode of regulation distinct from that of other Rho proteins and indeed GTPases in general, because their affinity for GDP is below detection levels, and they have very low or undetectable rates of GTP hydrolysis in vitro (Chardin, 1999). It is not known how Rnd proteins are regulated, although recent evidence shows that Ras can upregulate *RhoE/Rnd3* expression (Hansen et al., 2000).

Steps of cell migration

Cell migration can be divided into four mechanistically separate steps: lamellipodium extension, formation of new adhesions, cell body contraction, and tail detachment (Fig. 2; Lauffenburger and Horwitz, 1996). I will first consider the evidence linking specific Rho proteins to each of these steps and then go on to discuss other ways in which they contribute to cell migration.

Lamellipodium extension

Lamellipodium extension involves actin polymerization, and it is widely believed that lamellipodia consist of branching filament networks formed through the actin-nucleating activity of the Arp2/3 complex (Fig. 3; Pollard et al., 2000). Rac is required for lamellipodium extension induced by growth factors, cytokines and extracellular matrix components, and videomicroscopy experiments show that when Rac is inhibited cells cannot migrate (Allen et al., 1998; Nobes and Hall, 1999; Knight et al., 2000). A similar requirement for Rac is observed in Boyden chamber experiments, in which cells are plated on filters and the passage of cells through the filters is measured (Anand-Apte et al., 1997; Leng et al., 1999; Banyard et al., 2000). Effects of constitutively active Rac1 on migration vary, probably reflecting differences in cell type, stimulus, expression level and timecourse of expression. For example, constitutively active Rac1 inhibits growth-factor-induced macrophage migration, because lamellipodia extend all around the cells and they do not polarize (Allen et al., 1998). However, activated Rac1 can either promote or reduce translocation of cells across Boyden chambers, depending on the conditions (Leng et al., 1999; Banyard et al., 2000). Effects of overexpressing wild-type Rac1 on cell migration have not been extensively investigated, but in one study it was shown to increase PDGF-induced cell migration in Boyden chambers (Hooshmand-Rad et al., 1997).

Recent analysis of cells derived from Rac1- and Rac2-null mice supports a central role for Rac in cell migration. Rac2 is

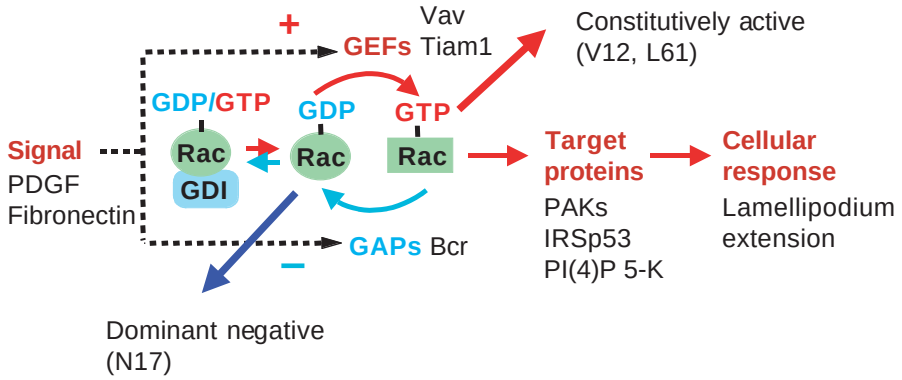

Fig. 1. Regulation of Rho family proteins. Most Rho proteins are active when bound to GTP, and inactive when bound to GDP. They are activated in response to extracellular signals including soluble cytokines, growth factors and extracellular matrix proteins. Activation is catalysed by exchange factors (GEFs) and inactivation by GTPase-activating proteins (GAPs). Several Rho family proteins also bind to guanine-nucleotide-dissociation inhibitors (GDIs) in the cytoplasm, and are inactive in this complex. Their activation therefore also depends on dissociation of GDI. When bound to GTP, they interact with target proteins to induce downstream responses.

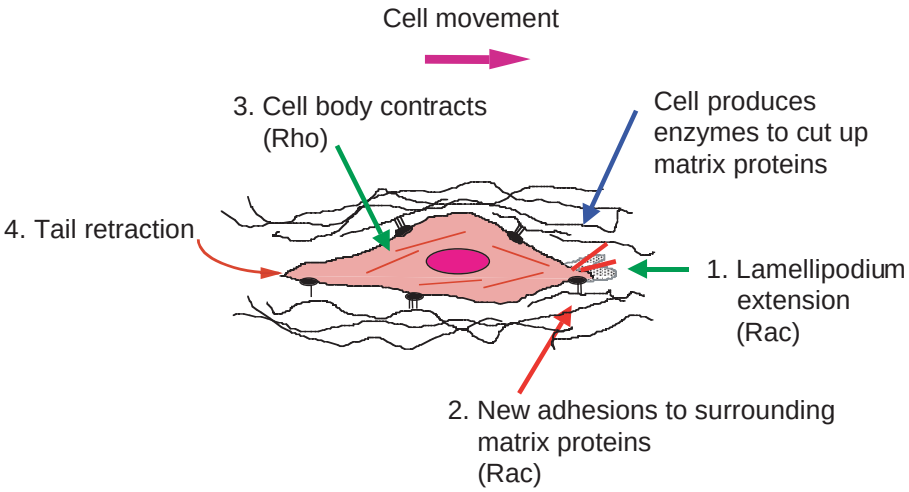

Fig. 2. A model for the steps of cell migration. A migrating cell extends a lamellipodium at the front. This extension is stabilized through the formation of new adhesions to the extracellular matrix. The cell body is moved forward by actomyosin-mediated contraction. Finally, the tail of the cell detaches from the substratum and retracts. Migrating cells also secrete proteases that cut up extracellular matrix proteins, and this is important for cell movement.

specifically expressed only in haematopoeitic cells, and neutrophils derived from mice lacking Rac2 show reduced migration speed in vitro as well as reduced F-actin polymerization in response to chemoattractants (Roberts et al., 1999). In addition, recruitment of neutrophils to inflammatory sites is much reduced in vivo. In contrast to Rac2, Rac1 is ubiquitously expressed, and Rac1-deficient embryos die before E9.5 (Sugihara et al., 1998). However, cultured epiblast cells derived from early embryos showed reduced migration rate (determined by videomicroscopy). Whether Rac3 is also important for cell migration remains to be determined, but interestingly Rac3 is highly expressed in the brain (Haataja et al., 1997; Malosio et al., 1997) and hyperactivated in some breast cancer cell lines (Mira et al., 2000). Rac proteins are also clearly required for cell migration in *Drosophila* and *C. elegans* (Reddien and Horvitz, 2000; Blelloch et al., 1999; Lu and Settleman, 1999), and Rac1 proteins in *Dictyostelium* have recently been shown to regulate pseudopod extension (equivalent to lamellipodia) and cell migration (Chung et al., 2000; Dumontier et al., 2000).

These studies all indicate that Rac is a key regulator of migration because of its ability to stimulate lamellipodium extension, but recent reports suggest that Rac can be bypassed in certain circumstances. For example, expression of active Rab5 (which regulates endocytosis) can induce lamellipodium extension independently of Rac (Spaargaren and Bos 1999), and membrane ruffling in immature dendritic cells does not require Rac (West et al., 2000). In colon carcinoma cells plated on laminin, dominant negative Rac does not inhibit membrane ruffling or cell spreading, and instead Rho is implicated in lamellipodium extension (O'Connor et al., 2000). In the latter case, Rho might link to the Arp2/3 complex, which is consistent with the observation that Rho and the Arp2/3 complex but not Rac/Cdc42 are required for integrin-mediated phagocytosis (May et al., 2000).

How does Rac get activated? Rac activation by both tyrosine kinases and G-protein-coupled receptors is often dependent on phosphoinositide 3-kinase (PI 3-kinase) activity, and inhibitors of PI 3-kinase block Rac activation (Sander et al., 1998; Rickert et al., 2000; Royal et al., 2000). In addition, the

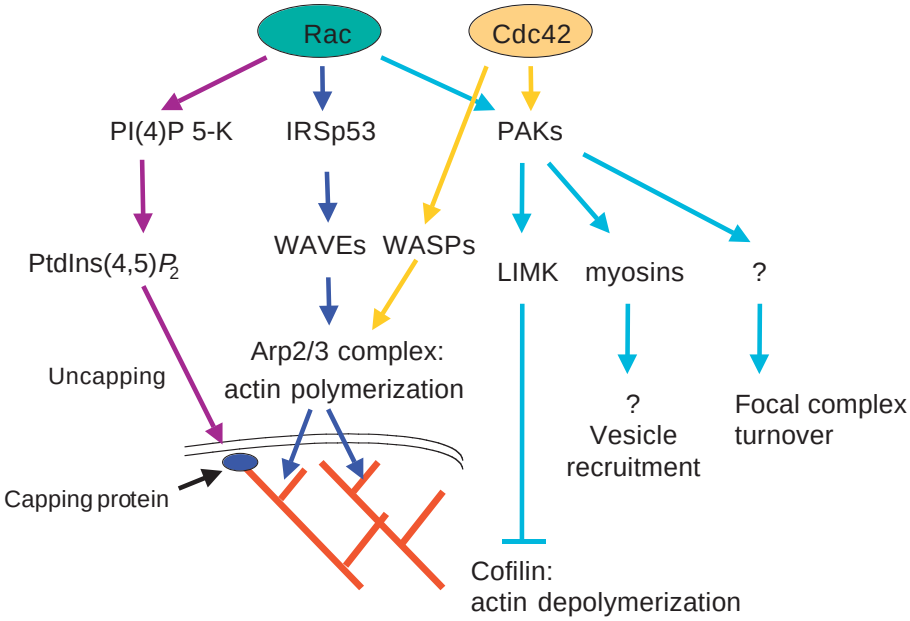

Fig. 3. A model for Rac-induced lamellipodium extension. Rac is postulated to act through several downstream targets to regulate F-actin accumulation at the leading edge of cells, in lamellipodia. It stimulates Arp2/3-complex-induced actin polymerization by interacting with a complex of IRSp53 and WAVE proteins. This leads to the formation of a branched filament network, because the Arp2/3 complex preferentially nucleates new actin filaments on the sides of existing filaments. Rac can also induce actin filament uncapping by generating phosphatidylinositol 4,5-bisphosphate locally, generating extra sites for actin polymerization. Finally, Rac acts via PAKs to stimulate LIMK, which inhibits cofilin-induced actin depolymerization, allowing increased accumulation of polymerized actin at the leading edge of cells. PAK may also contribute to migration in other ways by regulating myosin function and focal complex turnover. Crosstalk of Rac with Cdc42 via IRSp53 and/or PAKs may regulate the level of Rac signalling.

products of PI 3-kinase, PtdIns(3,4,5)P_3/PtdIns(3,4)P_2, appear to be enriched at the leading edge of migrating neutrophils and *Dictyostelium* (Meili et al., 1999; Haugh et al., 2000; Servant et al., 2000), which suggests that they activate Rac specifically here. Indeed, a novel technique for detecting where Rac is active in living cells has revealed that active Rac is localized preferentially towards the front of migrating cells (Kraynov et al., 2000). The PtdIns(3,4,5)P_3-responsive exchange factors at the leading edges of cells have not yet been identified, although the Rac exchange factors Tiam1 and Vav1 are likely candidates because they can be regulated by PI 3-kinase products (Das et al., 2000; Fleming et al., 2000). Another route to Rac activation has been delineated in *C. elegans* and *Drosophila*, in which genetic studies have revealed that Rac acts downstream of the Crk/DOCK180 adaptor proteins during developmental processes involving cell migration (Nolan et al., 1998; Reddien and Horvitz, 2000). Studies in mammalian cells have also shown the importance of Crk/DOCK180 together with Cas, another adaptor protein, for Rac activation and lamellipodium extension (Kiyokawa et al., 1998; Cheresh et al., 1999). Given that none of these proteins is an exchange factor for Rac, it is still not clear how this complex of proteins activates Rac, although Sos1 (an exchange factor for Ras and Rac) could be recruited through Crk (Cheresh et al., 1999). Sos1 is also implicated in linking Ras to Rac activation through two further adaptor proteins, Eps8 and E3b1 (Scita et al., 1999). Finally, Rac can be activated by integrin engagement (del Pozo et al., 2000), and Vav family exchange factors have been implicated in this process (Moores et al., 2000; Schwartz and Shattil, 2000). In the case of β3 integrins, Rac activation appears to depend on the initial formation of small Rac-independent integrin clusters and the action of the protease calpain (Bialkowska et al., 2000).

How does activated Rac coordinate lamellipodium extension? Several Rac targets are likely to be involved in this process (Fig. 3). First, Rac stimulates new actin polymerization, and one way it can do this is to stimulate the Arp2/3 complex, which initiates the formation of new actin filaments on the sides of existing filaments to form a branching actin filament network (Pollard et al., 2000). Recent evidence suggests that the Arp2/3 complex is activated by Rac through its target IRSp53 (also known as IRS-58; Fig. 3; Miki et al., 2000). Rac interacts with IRSp53, which in turn interacts through an SH3 domain with WAVE, which then binds to and activates the Arp2/3 complex (Fig. 3). There are three WAVE isoforms (also known as SCARs) so far identified, but WAVE2 seems to be the predominantly expressed isoform in fibroblasts (Miki et al., 2000). Interestingly, IRSp53 binds through a different domain to Cdc42 (Govind et al., 2001), so it could act as a direct link between Cdc42 and Rac, which would explain the observation that Cdc42 can induce Rac-dependent lamellipodium extension (Hall, 1998). IRSp53 can also bind to a Rho target, Dia1 (Fujiwara et al., 2000), and this might underlie the ability of Rho to contribute to lamellipodium extension in the cases described above.

In addition to activating the Arp2/3 complex, Rac can stimulate actin polymerization by promoting the uncapping of actin filaments at the plasma membrane. In resting cells, existing actin filaments are capped at their barbed (+) ends with capping proteins to prevent spontaneous actin polymerization. In platelets, Rac acts via a phosphatidylinositide 4-phosphate 5-kinase (PI*P* 5-kinase) to induce the formation of PtdIns(4,5)P_2, which then binds to capping proteins and removes them from the barbed ends of actin filaments (Carpenter et al., 1999; Tolias et al., 2000).

As well as inducing actin polymerization, Rac may affect the rate of actin depolymerization. Rac has been reported to stimulate the activity of LIM-kinase via the Rac/Cdc42 target PAK (Fig. 3; Edwards et al., 1999; Stanyon and Bernard, 1999). LIM-kinase then phosphorylates and inactivates cofilin, a protein that can promote actin depolymerization (Stanyon and Bernard, 1999). This suggests that Rac would inhibit cofilin-induced depolymerization. However, there is also strong evidence that cofilin is required for and promotes lamellipodium extension and cell migration (Aizawa et al., 1996; Chen et al., 2001), either by promoting release of actin monomers that can then be reincorporated into growing actin filaments at the plasma membrane and/or by severing actin filaments and thereby providing more barbed ends for actin polymerization (Chan et al., 2000; Zebda et al., 2000). The effect of Rac on cofilin activity therefore warrants further investigation.

A number of myosins have been implicated in cell migration (Mermall et al., 1998), and Rac can affect the phosphorylation of both myosin II heavy chain (MHC; van Leeuwen et al., 1999) and myosin light chain (MLC) via PAK (Daniels and Bokoch, 1999; Kiosses et al., 1999). Precisely how these effects of Rac on myosins could contribute to lamellipodium extension has not been established. On the one hand, MLC phosphorylation is enhanced in the lamellipodial region of cells (Matsumura et al., 1998), which suggests a role for myosins in lamellipodium extension, but on the other hand Rac-induced MHC phosphorylation correlates with loss of cortical MHC, which presumably leads to decreased cortical actomyosin tension and thereby cell spreading (van Leeuwen et al., 1999). To complicate the issue, expression of mutant PAKs can enhance or inhibit cell migration and have different effects on MLC phosphorylation depending on the cell type (Kiosses et al., 1999; Sells et al., 1999). Myosins also regulate membrane traffic (Mermall et al., 1998), and new membrane and proteins need to move to the front of migrating cells. In fact it has been suggested that receptors are selectively exocytosed at the leading edge of cells by a Rac-dependent mechanism (Bretscher and Aguado-Velasco, 1998), although a recent report has disputed this model (Bailly et al., 2000). Interestingly, myosins might also act upstream of Rac: a novel myosin in *Dictyostelium* has an exchange factor domain for Rac1, and can promote cell protrusion (Geissler et al., 2000). Clearly, Rac-mediated effects on myosins are important for lamellipodium extension, but further work is needed to determine which myosins are affected under specific conditions and whether they themselves feed back to regulate Rac activity.

Formation and turnover of new cell-substrate adhesions

Small focal complex structures are localized in the lamellipodia of most migrating cells, and are believed to be important in mediating the attachment of the extending lamellipodium to the extracellular matrix (Fig. 2; Lauffenburger and Horwitz, 1996). Rac is required for focal complex assembly (Nobes and Hall, 1995; Allen et al., 1997; Rottner et al., 1999), but whether this reflects an active Rac-

induced process or that focal complexes only form in lamellipodia is not clear. Cell adhesion to the extracellular matrix itself activates Rac and Cdc42: for example, plating cells on fibronectin induces Rac and Cdc42 activation, and this in turn is required for cell spreading (Price et al., 1998). It is therefore possible that continuous formation of new interactions between integrins and the extracellular matrix at the leading edge of cells maintains Rac active here and that this could provide a positive feedback loop allowing cells to carry on migrating even when receptor signalling is downregulating (Allen et al., 1998; Bailly et al., 2000). Rac-mediated recruitment of activated integrins to the leading edge of cells may also contribute to maintaining this cell migration feedback loop (Kiosses et al., 2001). The speed of cell migration is dependent on substrate composition, and indeed the relative levels of Rho, Rac and Cdc42 activation vary with extracellular matrix composition (Adams and Schwartz, 2000; Ridley, 2000; Wenk et al., 2000). There is thus continuous crosstalk between integrins and Rac to allow cells to respond to changing extracellular matrix composition (Schwartz and Shattil, 2000).

Focal complexes can be disassembled as the cell lamella moves over them, or in slowly migrating cells such as fibroblasts they can mature into Rho-induced focal adhesions (Rottner et al., 1999). In either case, it is important that focal complexes/adhesions turn over for cells to migrate: a high level of integrin-mediated adhesion inhibits cell migration because of the strength of attachment to the extracellular matrix, and this correlates with high levels of Rho activity (Cox et al., 2001). As well as being involved in focal complex formation in lamellipodia, Rac can induce focal complex/adhesion turnover both directly, through PAK (Zhao et al., 2000), and indirectly, by antagonizing Rho activation (Sander et al., 1999). PAK interacts with a complex of the exchange factor PIX, paxillin and a GIT family protein to localize to focal complexes/adhesions, and once there induces their disassembly, presumably by phosphorylating one or more focal adhesion components (Zhao et al., 2000). Interestingly, integrin-mediated adhesion appears to be required for growth-factor-activated Rac to couple to its downstream target PAK (del Pozo et al., 2000), which suggests that a critical level of integrin engagement triggers PAK activation and thus ultimately focal complex/adhesion disassembly. However, other signals, including Src and FAK, can also induce focal adhesion disassembly (Jones et al., 2000), and recently it has been suggested that microtubules target focal adhesions to deliver proteins that induce their breakdown (Kaverina et al., 1999).

Cell body contraction

Cell body contraction is dependent on actomyosin contractility (Mitchison and Cramer, 1996) and can be regulated by Rho. For example, when Rho is inhibited, macrophages continue to extend processes, but the cell body does not translocate significantly (Allen et al., 1997; Allen et al., 1998). Rho acts via ROCKs (also known as Rho-kinases) to affect MLC phosphorylation (Fig. 4), both by inhibiting MLC phosphatase and by phosphorylating MLC (Kaibuchi et al., 1999; Amano et al., 2000). MLC phosphorylation is also regulated by MLC kinase (MLCK), which is activated by calcium, and stimulated by the ERK MAPKs (Hansen et al., 2000). It is likely that ROCKs and MLCK act in concert to regulate different aspects of cell contractility, because ROCKs appear to be required for MLC phosphorylation associated with actin filaments in the cell body, whereas MLCK is required at the cell periphery (Totsukawa et al., 2000).

As in the case of PAK, the effect of inhibiting Rho on cell migration rate depends on the cell type, and this probably reflects the basal level of stress fibres and focal adhesions in cells. In cells that have stress fibres, such as cultured fibroblasts, the high level of substrate adhesion through stress-fibre-associated focal adhesions inhibits cell migration (Cox and Huttenlocher 1998; Cox et al., 2001). Reducing Rho activity therefore has two opposing effects: it promotes migration by lowering adhesion, but decreases cell migration by inhibiting cell body contraction. In less adherent cells that lack focal adhesions, such as macrophages, neutrophils and various cancer cell lines, Rho does not affect adhesion but induces cell body contraction, and here Rho and ROCK are clearly required for cell polarization and migration (Allen et al., 1998; Niggli, 1999; Wicki and Niggli, 2001). Rho activity therefore does not necessarily correlate with stress fibre levels, as illustrated by the observation that Ras-transformed fibroblasts have high levels of active Rho but no stress fibres

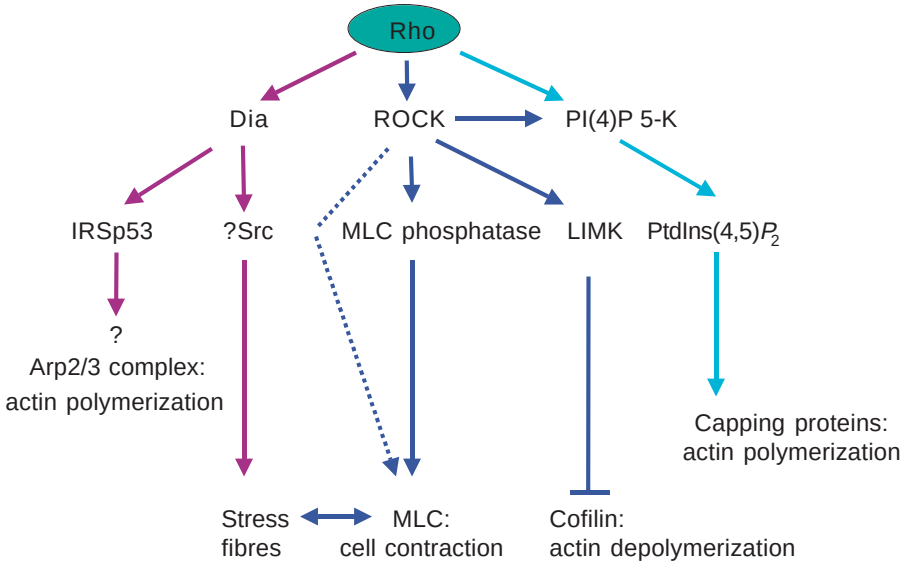

Fig. 4. Some targets for Rho linked to actin reorganization. Rho via ROCK can stimulate myosin light chain (MLC) phosphorylation through inactivation of MLC phosphatase and also probably through direct phosphorylation of MLC. ROCK together with Dia induces stress fibre formation. Dia is dependent on Src for its contribution to stress fibres and has also been reported to interact with IRSp53, which can mediate Arp2/3-complex-induced actin polymerization (see Fig. 3). ROCK can also phosphorylate a number of other target proteins that may contribute to actin reorganization, including LIMK, which inhibits cofilin-mediated actin depolymerization. Rho can also regulate the activity of PI (4)P 5-kinases (PI 5-K) to induce an increase in PtdIns(4,5)P_2 levels and thereby affect capping proteins, as indicated for Rac in Fig. 3.

(Sahai et al., 2001). It has been suggested that the lack of stress fibres reflects a Ras-induced decrease in the coupling of Rho to ROCK (Sahai et al., 2001). Alternatively, Ras could act via Raf to induce increased RhoE expression (Hansen et al., 2000), which can concomitantly reduce the level of stress fibres and enhance cell migration rate (Guasch et al., 1998).

In addition to MLC phosphorylation, Rho has other effects on the actin cytoskeleton that are relevant to cell migration (Fig. 4). Like PAK, ROCK activates LIMK to inhibit cofilin-mediated actin depolymerization, thereby promoting F-actin accumulation (Maekawa et al., 1999; Sumi et al., 1999). Another way for Rho to induce F-actin accumulation is via PI*P* 5-kinases (see above; Ren and Schwartz, 1998), and recently ROCK has been reported to stimulate PI*P* 5-kinase activity (Oude Weernink et al., 2000), although whether this involves direct phosphorylation of a PI*P* 5-kinase by ROCK is not known. Rho is also linked via Dia proteins to the actin cytoskeleton, and expressing constitutively active Dia together with ROCK effectively mimics Rho-induced stress fibre formation (Ridley 1999; Tominaga et al., 2000). Dia interacts directly with Src and is dependent on Src kinases for its effect on stress fibres (Tominaga et al., 2000). This is seemingly at odds with the ability of oncogenic Src to induce loss of focal adhesions (Jones et al., 2000), which would be expected to correlate with a decrease in stress fibres, but it is possible that what Dia really does is to act via Src to limit the size of ROCK-induced focal adhesions, thereby allowing the formation of many stress fibres rather than the few large ROCK-induced actin bundles. Dia proteins have also been reported to interact with IRSp53 (Fujiwara et al., 2000), which, as described above, can interact with WAVEs to induce actin polymerization, although so far whether Dia interaction with IRSp53 affects actin polymerization is not known.

Tail detachment

Tail detachment can often be the rate-limiting step of cell migration (Palecek et al., 1998). The mechanisms regulating tail detachment depend on the type of cell and strength of adhesion to the extracellular matrix (Cox and Huttenlocher, 1998; Palecek et al., 1998). In slowly moving cells tail detachment appears to depend on the action of the protease calpain, which degrades focal adhesion components at the rear of cells (Palecek et al., 1998; Glading et al., 2000). Interestingly, calpain activity can be regulated by ERK in EGF-stimulated cells (Glading et al., 2000), but so far there is no indication that it is regulated by Rho family members. A reduction in Rho activity could inhibit tail detachment, however, through decreased actomyosin contractility (Cox and Huttenlocher, 1998). Conversely, in cells such as fibroblasts that have large focal adhesions, inhibition of Rho would reduce adhesion and this could actually promote tail detachment. As described above, other signals, including Src, FAK and PAK, can induce focal adhesion disassembly (Jones et al., 2000; Zhao et al., 2000) and may contribute to tail detachment (Cox and Huttenlocher, 1998).

Other Rho-regulated cellular responses contributing to cell migration

Secretion of proteases: remodelling the environment

For cells to migrate in a three-dimensional matrix, secretion of matrix-degrading enzymes is crucial, and, even on a two-dimensional matrix, protease production can be important for migration (Murphy and Gavrilovic, 1999). Although the involvement of Rho GTPases in regulating the secretion and/or activation of secreted proteases has not been studied extensively, there are some indications that they could play a role. For example, constitutive expression of activated Rac and Cdc42 induces activation of JNK, which phosphorylates and activates the transcription factor Jun. Jun is a component of the AP-1 transcription factor complex and regulates transcription of many genes, including matrix metalloprotease (MMP) genes (Westermarck and Kahari, 1999). Rac is required for shear-stress-induced MMP9 expression in chondrocytes (Jin et al., 2000), and activated Rac can induce expression of the MMP1/MMP collagenase 1 in fibroblasts (Kheradmand et al., 1998). The long-term effects of expressing activated Rac/Cdc42 on cell migration therefore reflect both direct changes to the actin cytoskeleton and changes in gene transcription.

Filopodia: sensing the extracellular environment

In neuronal growth cones, filopodia are classically regarded as sensors for the extracellular milieu, steering the direction of growth cones during axon pathfinding (Kater and Rehder, 1995), and the role of Rho GTPases in regulating growth cone guidance has been recently reviewed (Dickson, 2001). Increasing evidence indicates that Cdc42, which regulates filopodium formation, is required for direction sensing during chemotaxis in other cell types as well as neurons (Allen et al., 1998; Nobes and Hall, 1999). By extending out from cells into the surroundings, receptors on filopodia could detect changes in extracellular signals that would then be transmitted back into cells. The role of Cdc42 in this process is presumably to initiate the actin polymerization required for filopodium extension, and indeed the ability of Cdc42 to stimulate actin polymerization via its interaction with WASp and N-WASp, leading to activation of the Arp2/3 complex, is well-characterized (Fig. 3). Interestingly, Cdc42 localizes predominantly to the Golgi complex, although it is also present on the plasma membrane (Erickson et al., 1996), and possibly on other intracellular vesicles (Michaelson et al., 2001). This suggests that Cdc42 has another, or overlapping, function in regulating vesicle trafficking (Wu et al., 2000; Ridley, 2001).

As well as Cdc42, its close relatives TC10 and TCL can interact with WASp proteins and thus have the potential to stimulate actin polymerization (Neudauer et al., 1998; Vignal et al., 2000). TC10 also induces filopodium extension (Neudauer et al., 1998), and it would be interesting to know how it affects directional cell migration. Interestingly, TC10 expression is upregulated following nerve injury, and it is able to promote neurite extension, which suggests that it may play an important role in growth cone extension (Tanabe et al., 2000). Similarly, RhoG can also promote neurite extension in PC12 cells (Katoh et al., 2000).

Microtubule dynamics

Although the majority of studies on Rho GTPases have concentrated on their roles in regulating the actin cytoskeleton, recent evidence has indicated that they can also affect the organization of microtubules (Waterman-Storer and Salmon, 1999). The extent of microtubule involvement in cell migration varies depending on the cell type. Some rapidly migrating cells,

such as neutrophils and keratocytes, are not affected by microtubule polymerization inhibitors (e.g. nocadazole), and fragments of keratocytes can migrate in the absence of microtubule-organizing centres. In contrast, nocadazole rapidly inhibits the migration of fibroblasts and epithelial cells (reviewed by Waterman-Storer and Salmon, 1999). Why are microtubules important for cell migration in some cells but not in others? Microtubules appear to be required for tail retraction (Ballestrem et al., 2000), which possibly reflects the fact that microtubules can target focal adhesions and induce their turnover in slow-moving cells (Kaverina et al., 1999; Kaverina et al., 2000). In this scenario, fast-moving cells such as neutrophils that do not have focal adhesions are not dependent on microtubule-mediated delivery of adhesion-disassembling proteins. The size of lamellipodial extensions may also contribute to the requirement for microtubules: larger extensions may rely more on microtubule infiltration to stabilize the extension.

How do Rho GTPases link with microtubules? First, microtubule depolymerization induced by nocadazole activates Rho (Liu et al., 1998), whereas washout of nocadazole activates Rac (Waterman-Storer et al., 1999). These effects could be mediated by changes in the activity of the several GEFs that have been reported to associate with microtubules (Ren et al., 1998; Glaven et al., 1999; van Horck et al., 2001). Second, Rho has been shown to be required for stabilization of microtubules induced by lysophosphatidic acid in fibroblasts (Cook et al., 1998; reviewed by Gundersen and Cook, 1999), and this may reflect a requirement for stable microtubules to counteract the contractility induced by Rho via ROCK (Pletjushkina et al., 2001). The Rho target Dia could provide a link between Rho and microtubules, because it regulates microtubule and F-actin polarization (Ishizaki et al., 2001). Cdc42 and Rac could also affect microtubule stability through stathmin, which can be phosphorylated by PAK (Daub et al., 2001). PAK phosphorylation of stathmin would inhibit its action in destabilizing microtubules, and this could be important for ensuring that microtubules grow into the lamellal region of migrating cells (Waterman-Storer and Salmon, 1999).

Cdc42 can mediate the polarization of the microtubule network observed in migrating cells (Nobes and Hall, 1999). Cdc42 may be directly linked to the microtubule network via its target CIP4, which interacts with both WASp and microtubules (Linder et al., 2000; Tian et al., 2000). Interestingly, microtubules, Cdc42 and WASp are required for the formation of podosomes, which are unique, rapidly turning over adhesion structures found at the leading edge of dendritic cells and macrophages (Linder et al., 1999; Linder et al., 2000), although whether Cdc42 is involved in linking microtubules to podosomes is not known. A recently identified target for Cdc42, PAR6, is involved in generating cell polarity and asymmetric cell division in *C. elegans* (Kim, 2000), and it will therefore be interesting to see whether Cdc42 acts via PAR6 to induce microtubule polarization.

Intermediate filaments

As in the case of microtubules, the role of intermediate filaments in cell migration depends on the circumstances. For example, although lack of vimentin has no discernable effect on mouse development, cultured fibroblasts derived from vimentin-null mice show impaired migration and contraction in collagen gels (Eckes et al., 1998), and the mice show impaired wound healing, owing to a failure of fibroblast migration into the wound site and subsequent mesenchymal contraction (Eckes et al., 2000). Intermediate filament protein phosphorylation is important for regulating the intermediate filament network, both during interphase and at cytokinesis (Herrmann and Aebi, 2000). Expression of activated RhoA induces reorganization of vimentin filaments (Paterson et al., 1990; Sin et al., 1998), and ROCK phosphorylates vimentin (Goto et al., 1998; Sin et al., 1998) and the intermediate filament protein GFAP (Kosako et al., 1997; Matsuzawa et al., 1997). These effects of ROCK have been linked to reorganization of intermediate filaments at cytokinesis (Herrmann and Aebi, 2000), and whether there is also a link with cell migration is not yet known. Activated RhoG, Rac and Cdc42 induce collapse of vimentin filaments into the perinuclear region, as do growth factors such as PDGF and bradykinin (Valgeirsdottir et al., 1998; Meriane et al., 2000), and this response could be important for remodelling the intermediate filament network during cell migration.

Conclusions

Rho GTPases coordinately regulate multiple aspects of cell migration, affecting the different components of the cytoskeleton as well as cell-substrate adhesion and possibly matrix remodelling. However, we still have a long way to go in defining how Rho, Rac and Cdc42 fit into the global picture of signalling during cell migration. It is relatively easy to perturb cell migration by altering the flux down multiple intracellular signalling pathways, and it is important to remember that many of the dominant negative or constitutively active constructs we introduce into cells or the inhibitors we use are not absolutely specific for one protein. In addition, the contribution of particular signalling proteins will vary depending on cell type and circumstances, and thus it is not always straightforward to extrapolate from one system to another. Studies of model organisms have contributed key information on the roles of specific Rho family proteins in cell migration, but *Drosophila*, *C. elegans* and *Dictyostelium* do not have the full complement of mammalian Rho subfamilies or the genetic diversity within subfamilies. In the future more studies on the less well-known or newly discovered family members (Table 1) will no doubt throw up many surprises and change our models radically. This will be essential if we are to gain a full understanding of how these proteins contribute to cell migration in development and in disease.

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