Rho: theme and variations

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In addition to their roles in organizing the actin cytoskeleton, members of the Rho family of GTP-binding proteins have recently been implicated in a plethora of other functions, including the activation of kinase cascades and transcription factors, and the control of endocytosis and secretion. Alongside this expansion in proposed functions has been the identification of multiple target proteins that interact directly with Rho, Rac or Cdc42. Molecular connections are now being made along the signalling pathways activated by members of the Rho family.

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Introduction

It is eleven years since the first gene encoding a Rho protein was cloned from *Aplysia*. Since then, eleven genes encoding mammalian Rho family members have been identified, and many homologous genes have also been isolated from other species, from yeast to *Drosophila* and *Dictyostelium* (Table 1) [1–7]. All of the proteins encoded by these genes belong to the superfamily of Ras-related GTP-binding proteins. In general, members of the superfamily cycle between a GTP-bound and a GDP-bound form, and hydrolyse GTP [1]. The level of active, GTP-bound protein is regulated by guanine-nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine-nucleotide dissociation inhibitors (GDIs) [8,9]. The alternation between GTP-bound and GDP-bound forms is thought to allow members of the Ras superfamily to act as bimodal switches, and they regulate many intracellular signalling pathways.

Early studies with Rho and Rac showed that they play major roles in regulating remodelling of the actin cytoskeleton induced by extracellular signals [1]. In addition, Rac was found to be an essential activator of the NADPH oxidase in phagocytes [10]. In the past two years or so, however, research on the Rho family has grown exponentially, identifying many potential protein targets and regulators, and revealing many apparently diverse functions for the members of the family in regulating a wide array of cellular responses — from endocytosis and secretion to transcriptional regulation and growth control. Several recent reviews have covered aspects of this field [9,11–13]; this review is an attempt to assimilate some of the latest exciting developments, focussing on the roles of Rho family proteins in mammalian cells.

The Rho family and the cytoskeleton

It is now well established that three members of the Rho family — Rho, Rac and Cdc42 — are involved in regulating the organization of the actin cytoskeleton. In fibroblasts, it has been shown that Rho regulates the formation of actin stress fibres, whereas Rac regulates lamellipodium formation and membrane ruffling, and Cdc42 regulates filopodium formation [1,11]. Rho is also required for the formation and maintenance of focal adhesions, the sites at which stress fibres are linked via integrins to the extracellular matrix [14], whereas Rac and Cdc42 regulate the formation of smaller ‘focal complex’ structures associated with lamellipodia and filopodia [11]. Cell motility involves the coordinated extension of lamellipodia and filopodia at the leading edge of the cell, together with contraction of the cell body and detachment of the rear edge, so Rho,

Table 1

Rho family members.

	Mammalian	*S. cerevisiae*	*S. pombe*	*Drosophila*	*Dictyostelium*
Rho-like	Rho (A, B, C)	Rho1		Rho1	
Rac-like	Rac (1, 2)			DRac (1,2)	Rac (1,2,3,B)
Cdc42-like	Cdc42, G25K	Cdc42	Cdc42	DCdc42	
Other	RhoD RhoE RhoG TC10 TTF	Rho2 Rho3 Rho4			RacA RacC RacD RacE

Members of the Rho family, identified in the species shown, are listed in groups according to their closest homology with the mammalian proteins. Apart from Rho, Rac and Cdc42, clear homologues of the mammalian proteins have not been identified in other species. Note that Rho family proteins have also been identified in several species that are not included here.

Rac and Cdc42 could play a crucial role in this process [15]. Indeed, there is some evidence that Rho and/or Rac are required for motile responses [16–18]. Rho family proteins have also been implicated in the formation of the actin-based contractile ring at cell division [7,9]. In addition, in the budding yeast *Saccharomyces cerevisiae*, Cdc42 and Rho1 are involved in regulating polarization of the actin cytoskeleton during budding [9]. Until recently, however, very little was known of the signal transduction pathways acting downstream of these proteins, leading to actin reorganization.

Rho and the actin cytoskeleton

A flurry of recent reports has established a model for a direct molecular pathway connecting Rho and the actin cytoskeleton. The key component of this pathway is ROKα/Rho-kinase, one of the recently identified Rho-interacting proteins (Table 2). ROKα/Rho-kinase and the closely related protein ROKβ/p160 are members of a growing family of proteins that includes myotonic dystrophy kinase [19–22]. The sequences reported for Rho-kinase [19] and ROKα [22] differ only at their amino termini, where Rho-kinase is nine amino acids longer. ROKα has been found to stimulate the formation of stress fibres and focal adhesions when overexpressed in HeLa cells [22]. The Rho-binding domain of ROKα is not required for this response, nor is the response affected by the presence of the Rho inhibitor C3 transferase (an exoenzyme from *Clostridium botulinum* which inactivates Rho by ADP-ribosylating it). Additionally, a 'kinase-dead' form of ROKα acts as a dominant-negative inhibitor of Rho function, inducing disassembly of stress fibres and focal adhesions. These results strongly suggest that ROKα lies downstream of Rho in the pathway leading to stress fibre formation; indeed, Rho can modestly stimulate the kinase activity of ROKα/Rho-kinase *in vitro* [19,20].

Table 2

Interacting 'targets' for Rho family proteins in mammalian cells.

	'Target'	Rho family interactions	Proposed function *in vivo*
Protein kinases	PKN	Rho	?
	ROK/Rho-kinase/p160	Rho	Actin reorganization
	PAK family	Rac/Cdc42	SAPK/p38 kinase activation
	$p70^{S6K}$	Rac/Cdc42	Mitogenic signalling
	$p120^{ACK}$	Cdc42	?
Phosphatases	MLC phosphatase (MBS)	Rho	Actin reorganization
Adaptor proteins	Rhophilin	Rho	?
	Citron	Rho	?
	$p67^{phox}$	Rac	NADPH oxidase
	WASP	Cdc42	Actin reorganization
Lipid kinases	PIP 5-kinase	Rho/Rac	Actin reorganization/ secretion
	PI 3-kinase	Rac/Cdc42	Actin reorganization/ mitogenic signalling

These proteins have been shown to interact specifically with GTP-bound Rho, Rac and/or Cdc42 in the yeast two-hybrid system, by affinity chromatography, and/or by overlay assay. The table does not include the many GAPs for Rho family proteins, which may also have effector functions [9].

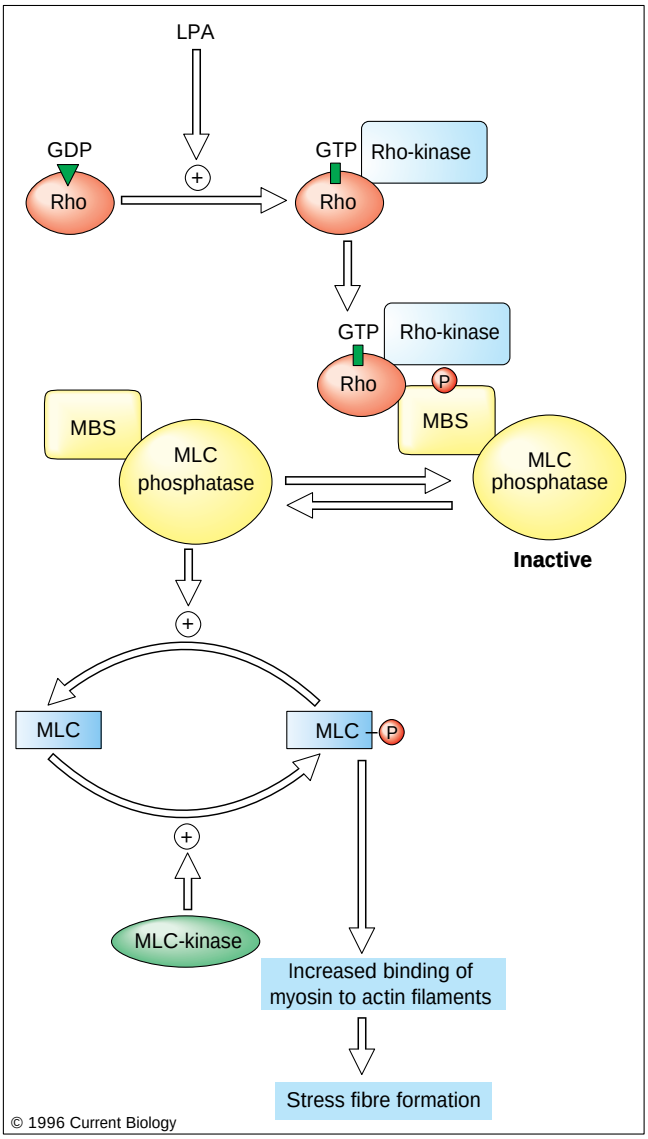
A molecular link between ROKα/Rho-kinase and the actin cytoskeleton is suggested by the observation that the myosin-binding subunit (MBS) of myosin light-chain (MLC) phosphatase is a good substrate *in vitro* for Rho-kinase [19]. In fact, Rho itself binds to MBS and stimulates the phosphorylation of MBS by Rho-kinase, thereby inhibiting MLC phosphatase activity *in vitro* [23]. In fibroblasts overexpressing RhoA, there is an increase in the phosphorylation both of MBS and of MLC itself. Phosphorylation of MLC is known to induce a conformational change in myosin II which increases its interaction with actin and enables the formation of myosin filaments, observed as a characteristic periodic pattern of myosin staining along stress fibres [24–26]. Taken together, therefore, these data suggest a model in which Rho induces stress fibre formation by increasing the phosphorylation of MLC, thereby stimulating the interaction of myosin with actin filaments (Fig. 1).

Data accumulated by many laboratories over a number of years serve to reinforce this model centred on the phosphorylation state of MLC. For example, eight years ago it was found that injection into fibroblasts of antibodies raised against MLC kinase induced a decrease in MLC phosphorylation accompanied by the loss of stress fibres [27]. Lysophosphatidic acid (LPA) rapidly stimulates MLC phosphorylation, and various inhibitors of MLC kinase have been reported to prevent LPA- and Rho-induced contractility and stress-fibre formation [26,28]. Furthermore, it has been shown that Rho is involved in regulating smooth-muscle cell contraction, a process dependent on MLC phosphorylation [29]. The ability of Rho to modulate MLC phosphorylation could explain its involvement in cytokinesis, a process dependent on contraction mediated by myosin II and actin [9].

From the results described above, it is possible to conclude that Rho could induce the formation of contractile stress fibres primarily by reorganizing existing actin filaments, rather than by stimulating polymerization of actin. In mast cells, however, Rho can stimulate new actin polymerization [30]; Rho is also required for actin polymerization occurring during *Shigella* entry into HeLa cells [31]. In reality, there is likely to be a balance between reorganization and new polymerization, and the relative contribution of each may depend on the type of response involved.

A link between Rho and actin polymerization may be provided by phosphatidylinositol 4-phosphate 5-kinase (PIP 5-kinase), which generates phosphatidylinositol 4,5-bisphosphate (PIP_2). Although so far no PIP 5-kinase isoforms have turned up in screens for Rho-interacting proteins, Rho affinity columns can retain PIP 5-kinase, and PIP 5-kinase immunoprecipitates contain Rho [32]. In one report, however, PIP 5-kinase activity was not found to bind significantly to Rho affinity columns [33], suggesting that the interaction between PIP 5-kinase and Rho may be indirect, and thus its detection may be dependent on experimental conditions. Rho appears to act upstream of PIP 5-kinase, as it stimulates PIP 5-kinase activity in cell extracts [34]. As PIP_2 can bind to, and affect the activities of, several actin-binding proteins, it is possible that, by increasing the level of PIP_2, Rho could induce actin polymerization [11,13]. Interestingly, injection of anti-PIP_2

Figure 1



A molecular model for Rho-induced stress fibre formation. Lysophosphatidic acid (LPA) or another extracellular factor stimulates an increase in Rho–GTP. This interacts with Rho-kinase and with the myosin-binding subunit (MBS) of myosin light chain (MLC) phosphatase. Rho–GTP activates Rho-kinase, which phosphorylates MBS, leading to inactivation of the phosphatase. The phosphorylated form of MLC therefore accumulates, and this leads to increased binding of myosin to actin filaments and subsequently the formation of stress fibres. Note that it is not known whether Rho can interact simultaneously with Rho-kinase and MBS.

antibodies into fibroblasts leads to the loss of stress fibres [35], although the specificity of such antibodies *in vivo* is difficult to ascertain.

Rho and focal adhesions

In addition to stimulating the formation of stress fibres, Rho induces focal adhesion formation and stimulates the tyrosine phosphorylation of a number of focal adhesion proteins, including pp125^{FAK}, p130 and paxillin [1,36]. So far, no tyrosine kinase has yet been identified as directly interacting with Rho (Table 2). Instead, it is likely that Rho-induced protein-tyrosine phosphorylation is indirect and is mediated by one of the other Rho targets. One possible model for focal adhesion formation (Fig. 2) is that Rho-mediated reorganization of actin filaments into contractile stress fibres induces the clustering of integrins, thereby catalysing the formation of focal adhesions and the accompanying tyrosine phosphorylation of focal adhesion proteins. Indeed, actin reorganization precedes protein-tyrosine phosphorylation when activated Rho is introduced into cells [36], and LPA-induced protein-tyrosine phosphorylation is prevented by inhibitors of MLC kinase [26]. There is also evidence, however, that stress fibre formation and focal adhesion formation may be separate, but coordinated, events [11], and may therefore each be regulated by a different downstream target of Rho.

Rac/Cdc42 and the actin cytoskeleton

The best-characterized target proteins for Rac and Cdc42 are the p21-activated kinase (PAK) family of serine/threonine kinases ([37] and references therein). Work on these kinases has concentrated on their involvement in regulating cascades of other kinases that lead to transcription factor activation (see below), but so far there has been no definitive evidence to support or eliminate a role for them in regulating cytoskeletal responses induced by Rac or Cdc42.

As is the case for Rho, a potential link between Rac and increased actin polymerization involves PIP_2 generation. Rac affinity columns can bind PIP 5-kinase [33], and Rac can stimulate PIP_2 production in permeabilized platelets [38]. In this system, Rac and polyphosphoinositides (including PIP_2) induce uncapping of actin filaments, which in intact cells would normally precede actin polymerization. Hartwig *et al.* [38] therefore postulate that Rac-induced PIP_2 generation would lead to actin polymerization. The effect of Rho on PIP_2 production in permeabilized platelets was not reported, but in this context it is interesting to note that in experiments using fibroblast cell extracts, Rho, but not Rac, was reported to stimulate PIP 5-kinase activity [34]. The apparent discrepancy between these results may reflect the involvement of distinct isoforms of PIP 5-kinase responsive to Rac or Rho. It would therefore be of great interest to investigate the actions of Rho and other Rho family proteins, especially Cdc42, in platelets.

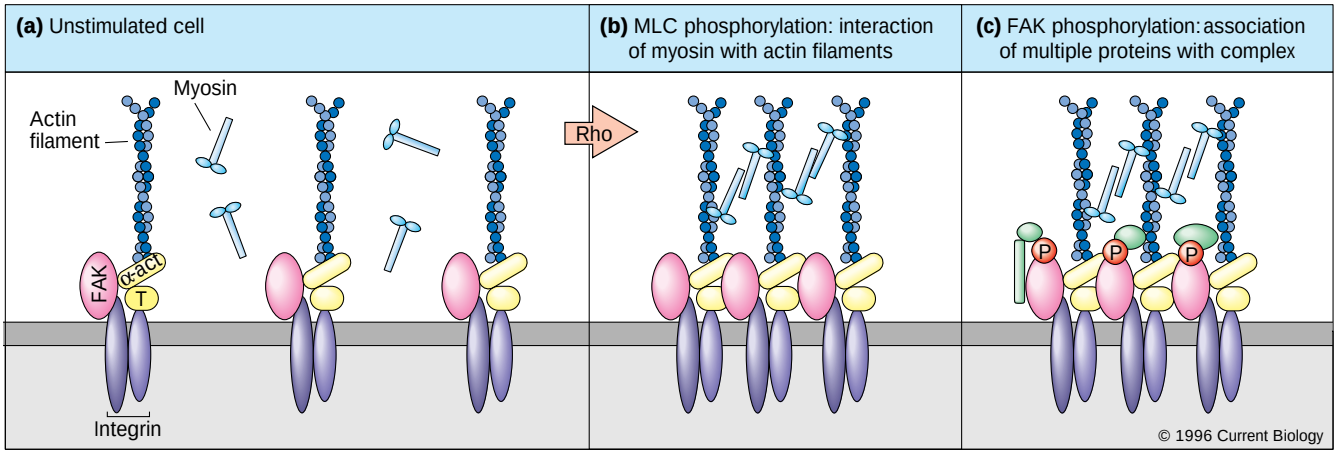
A specific connection between Cdc42 and the actin cytoskeleton has recently been suggested by studies on the Wiskott-Aldrich syndrome (WAS) protein (WASP) [39]. The *WAS* gene was initially isolated by positional cloning and shown to be mutated in WAS patients. It has subsequently been isolated as encoding a protein that interacts with Cdc42 and weakly with Rac. WASP expression is restricted to haematopoietic cells [40]. Although WASP has not been shown to localize to filopodia or affect filopodium formation, it does induce an unusual form of actin reorganization. When overexpressed in several cell types, including Jurkat cells (which normally express WASP), it is found to co-localize with clusters of polymerized actin [40] This effect is inhibited by co-expression of dominant-negative Cdc42, but not Rac or Rho. These results indicate that there is an interaction between WASP and the actin cytoskeleton involving Cdc42, but a direct role of WASP in Cdc42-induced filopodium formation has yet to be established. Interestingly, WASP also binds to c-Src family tyrosine kinases [41], which are known to regulate actin cytoskeletal organization [42] and may therefore be involved in Cdc42 signalling.

So far there has been little evidence that GAPs for Rho family proteins might function as effector proteins, in addition to acting as down-regulators. It appears, however, that *n*-chimaerin, a Rac/Cdc42 GAP, may play a role in Rac/Cdc42-mediated actin reorganization [43]. Microinjection of the *n*-chimaerin GAP domain alone inhibits Rac-mediated lamellipodium formation in fibroblasts, in line with previous observations on GAP domains [1]. In contrast, microinjection of the full-length protein induces the formation of both filopodia and lamellipodia, and these responses are dependent on Cdc42 and Rac proteins, respectively. The Cdc42- or Rac-binding domain of *n*-chimaerin is essential for these responses, suggesting that it interacts productively with Rac and Cdc42 to mediate downstream signalling. Interestingly, *n*-chimaerin also has a phorbol-ester-binding domain, and one possibility is that *n*-chimaerin acts by assisting the correct localization of Rac and Cdc42 to the plasma membrane through its ability to bind phospholipids.

Rho family proteins and growth control

There is accumulating evidence that Rho family proteins not only regulate cell morphology and actin organization, but also interact with a number of proteins regulating cell growth and mitogenesis. The initial suggestion that the Rho family might play a role in growth control came from the isolation of a number of oncogenes which encode exchange factors for Rho family members [9]. More recently, Rac and Rho have been shown to have transforming ability in some cell types, and to be required for Ras-induced transformation of fibroblasts [12]. Rac is also required for v-Abl to activate a mitogenic program [44]. In addition, it has been reported that microinjection

Figure 2



A hypothetical model for Rho-induced focal adhesion formation. In unstimulated cells **(a)**, integrins are not extensively clustered, but are associated with various proteins, including for example pp125^{FAK} (FAK), talin (T), and α-actinin (α-act). They are thereby associated with short actin filaments, but myosin is not associated with these filaments. Following activation of Rho **(b)**, for example by LPA, myosin light chain (MLC) becomes phosphorylated and myosin interacts productively with actin filaments, leading to the formation of contractile stress fibres. These fibres pull integrins and their associated proteins into clusters within the plasma membrane. Clustering of integrins leads to the phosphorylation of associated proteins **(c)**, for example pp125^{FAK}, which can autophosphorylate itself on tyrosine residues. This allows the association of many other signalling and structural proteins with the complex, forming focal adhesions and leading to downstream signalling.

of Rho, Rac or Cdc42 proteins into Swiss 3T3 fibroblasts stimulates DNA synthesis, and that inhibitors of these proteins block growth-factor-induced DNA synthesis [45]. The pathways used by these proteins to induce DNA synthesis are not clear, but there are several possibilities.

Rho and growth control

One mechanism whereby Rho could induce a mitogenic response in cells is through its ability to activate the transcription factor SRF, which binds to the serum response element (SRE) found in the promotors of a number of 'early' genes induced by growth factors [46]. Expression of Rho, Rac or Cdc42 stimulates the activity of SRF, but only Rho is required for serum- and LPA-induced SRF activation. The signalling pathway linking Rho to SRF has not been delineated, and it is possible that any of the proteins which interact with GTP-bound Rho could be involved (Table 2). Interestingly, the *S. cerevisiae* homologue of Rho, Rho1p, has been shown to interact directly with Pkc1p, a yeast protein kinase C homologue, leading to activation of a mitogen-activated protein (MAP) kinase cascade [47]. Rho does not activate any of the known MAP kinase cascades in mammalian cells [46,48,49], but it may activate a related pathway, perhaps involving the Rho-interacting kinase PKN.

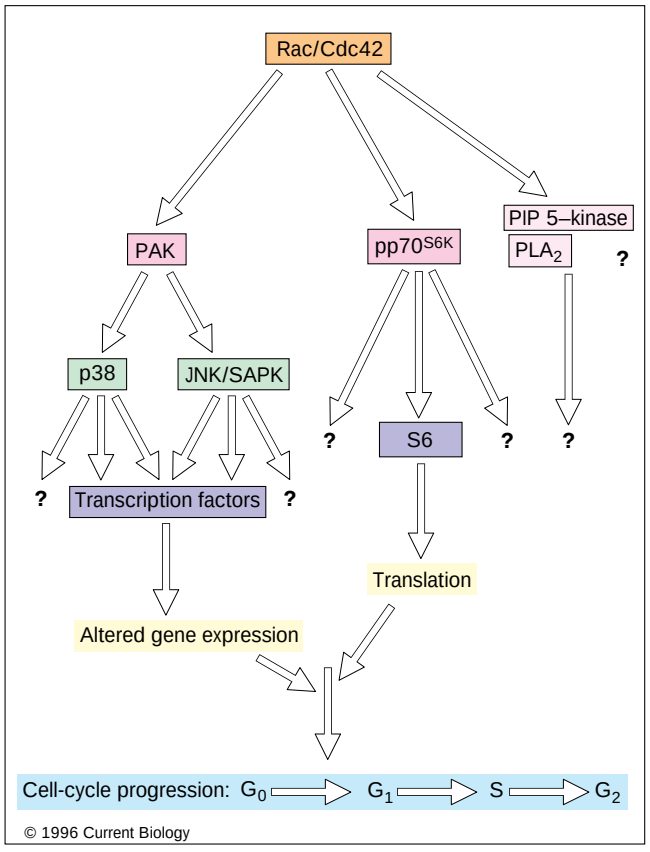
Rac/Cdc42 and growth control

The last year has seen an explosion of studies of the roles of Rac and Cdc42 in the activation of kinase cascades that could potentially be involved in mitogenic responses. It has been known for a while that Rac and Cdc42 interact with and activate the PAK family of serine/threonine kinases. A yeast homologue of PAK, Ste20, acts downstream of Cdc42 in the MAP kinase pathway activated by pheromones [50]. Several reports have now established links in mammalian cells between Rac/Cdc42, PAKs and the so-called 'stress-activated' protein kinase cascades, leading to activation of the stress-activated protein kinases (SAPKs, also known as Jun N-terminal kinases, or JNKs) and p38 kinases [51]. Using transient transfection assays, it has been shown that expression of activated forms of Rac and Cdc42 leads to increased SAPK and p38 kinase activity, and that dominant-negative Rac/Cdc42 mutants inhibit the activation of these kinases by some extracellular factors [45,48,49,52]. Furthermore, overexpression of PAK1 leads to activation of the p38 kinase [53], and expression of a constitutively active PAK stimulates SAPK activity ([37,52]. In all of these experiments, little or no activation of p42/p44 MAP kinases was observed, in contrast to the strong stimulation of MAP kinases by Ras.

Whether Rac/Cdc42-mediated activation of SAPK and p38 kinase can contribute to mitogenic stimulation of cells is unclear. These kinases are normally activated most potently by 'stress' stimuli which do not normally stimulate DNA synthesis [51]. But SAPK and p38 kinase are also weakly activated by some growth factors, and several transcription factors have recently been identified as targets for these kinases [54]. It is therefore possible that they can, in combination with other signals, contribute to mitogenesis by inducing expression of genes required for cell-cycle progression (Fig. 3).

Although this model is attractive, it is important to note that all of the data on the activation of these kinase pathways by Rac and Cdc42 come from long-term transient transfection assays. What is still lacking is direct evidence that Rac or Cdc42 can rapidly activate the kinases upon introduction into cells, in the same way as Ras has been shown to activate p42/p44 MAP kinases [55]. In addition, although platelet-derived growth factor (PDGF) has been reported to stimulate GTP loading on Rac, it is not yet known whether Rac or Cdc42 is similarly activated by stress stimuli such as tumour necrosis factor-α (TNF-α). In fact, agents that rapidly induce Rac-dependent ruffling/lamellipodia, such as PDGF, are only at best very weak activators of SAPK and p38 kinase, whereas TNF-α, which potently stimulates these kinases, does not detectably induce membrane ruffling in fibroblasts [51,56]. It is therefore possible that the effects observed in transient transfection assays reflect an indirect role of Rac and Cdc42 in producing or modulating signals leading to a stress response. Alternatively, stress signals may normally act directly through a different member of the Rho family, but Rac and Cdc42 are able to induce the same effects when overexpressed.

Figure 3



Downstream signalling pathways activated by Rac and Cdc42 which may lead to mitogenesis.

A second link to mitogenic signalling pathways was revealed recently when it was reported that Rac and Cdc42 interact with and activate pp70^{S6K} both *in vitro* and *in vivo* [57]. This kinase plays an important role in the progression of cells from G1 to S phase of the cell cycle, and is generally activated by mitogenic stimuli within minutes [58]. In transient transfections, activated Rac and Cdc42 induce an increase in the activity of co-transfected pp70^{S6K}, whereas dominant-negative forms of Rac and Cdc42 inhibit growth factor-induced activation of pp70^{S6K}. Neither SAPK nor p38 kinase appears to mediate the effects of Rac and Cdc42. Instead, GTP-bound Rac and Cdc42 can directly and specifically interact with pp70^{S6K} *in vitro*, and they co-immunoprecipitate from cells. How Rac and Cdc42 activate pp70^{S6K} in cells is unclear, as they do not stimulate its kinase activity *in vitro*. It would be of interest to determine whether phosphoinositide (PI) 3-kinase is also present in a complex with Rac/Cdc42 and pp70^{S6K}, as Rac and Cdc42 can both interact with PI 3-kinase [33,59], and PI 3-kinase is a key upstream regulator of pp70^{S6K} [58]. Perhaps Rac and Cdc42 act to facilitate interactions between pp70^{S6K} and its direct activators.

Finally, it has been reported that Rac is required for arachidonic acid release induced by epidermal growth factor (EGF), and that it also mediates calcium influx in response to EGF but not to all growth factors [60,61]. The signalling pathways linking Rac to these responses remain to be elucidated, but it is likely that they are important during mitogenic stimulation (Fig. 3).

Other responses regulated by the Rho family

The requirement for Rac in regulating the NADPH oxidase of phagocytes has been known for several years — as long as Rac's involvement in actin reorganization [10]. More recently, however, several other functions have been attributed to the Rho family, and these effects are apparently independent of their effects on actin reorganization. For example, Rac and Rho have been shown to regulate secretion in permeabilized mast cells. Recombinant Rac and Rho proteins enhance secretion, whereas C3 transferase and dominant-negative Rac inhibit secretion induced by the non-hydrolysable GTP analogue GTPγS [62]. Independently, Rac was purified from mast cells as a factor which could enhance secretion [63]. Comparison of the actin reorganization responses to Rac and Rho with the secretory responses shows that although actin reorganization is closely linked to secretion, the two responses are independently regulated [64].

Rho and Rac have also been implicated in the regulation of endocytosis [65,66]. In *Xenopus* oocytes, Rho has been found to enhance constitutive endocytosis/pinocytosis, whereas C3 transferase inhibits the process [66]. Conversely, in mammalian cells and in a cell-free endocytosis system, Rho and Rac inhibit receptor-mediated endocytosis

of clathrin-coated vesicles [65]. As is the case for secretion, these effects are apparently not mediated by changes in actin organization. Interestingly, one of the three closely related Rho proteins, RhoB, is localized to early endosomes, suggesting a role in endocytosis [67].

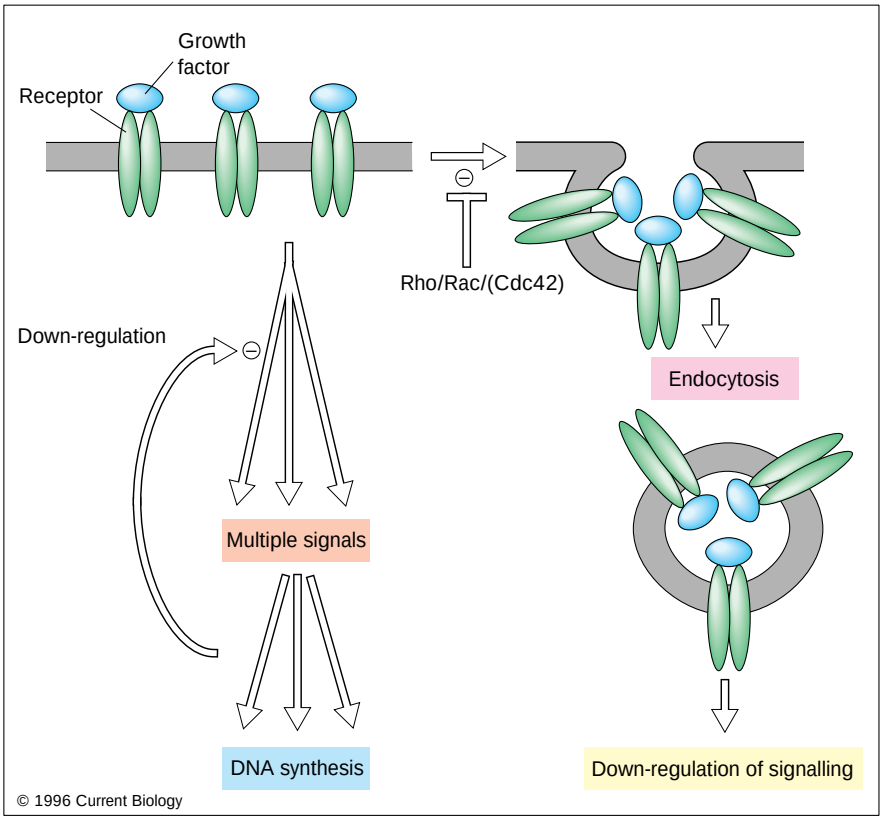
Is there a common mechanism underlying the effects of Rho and Rac on secretion and endocytosis? It is possible that their involvement in these processes reflects their ability to alter membrane phospholipid composition, for example through activation of PIP 5-kinase, PI 3-kinase or phospholipase D [68]. Indeed, it is becoming increasingly clear that phosphoinositides play an important role in regulating vesicle traffic [69], and there is evidence that PIP 5-kinase and phospholipase D are involved in secretion [68,69]. A comprehensive assessment of the effects of other Rho-related proteins, in particular Cdc42, on secretion and endocytosis would provide considerably more information on the specificity of these responses.

The inhibitory effects of Rho and Rac on receptor-mediated endocytosis suggest one interpretation of the mitogenic effects of Rho family proteins (Fig. 4). If receptor-mediated endocytosis is inhibited in cells over-expressing Rho or Rac (Cdc42 remains to be tested), then perhaps growth factor receptors that would normally be down-regulated by endocytosis instead stay for longer periods on the cell surface. Cells would then be sensitized to, or show prolonged responses to, growth factors for which the receptors are normally down-regulated by endocytosis, and thereby show an increased response to those factors; the growth factors might be present in serum or as autocrine factors. It has been argued that, because little or no p42/p44 MAP kinase activation is observed in Rac/Cdc42-transfected cells, Rac and Cdc42 do not act non-specifically to up-regulate signalling. But there may be tighter regulation of p42/p44 MAP kinase activity than of some signalling pathways, and even a transient activation of p42/p44 MAP kinases may be sufficient to induce a mitogenic response [70]. It should be possible to test the model shown in Figure 4, for example by investigating the levels of various receptors on the cell surface and their ability to be internalized.

New Rho family members – new modes of action?

Recently, two new members of the Rho family of proteins, RhoE and TTF, have been characterized [2,3]. Although their biological functions have not been elucidated, both proteins have some unusual features which suggest that they may act in fundamentally different ways to other members of the Ras superfamily. In RhoE, three amino

Figure 4



A model for the regulation of mitogenic signalling by Rho family proteins. Upon growth factor binding, receptors activate multiple pathways, ultimately leading to DNA synthesis. Rho family proteins may be directly involved in activating some of these pathways. Following ligand binding, many receptors cluster into clathrin-coated pits and are endocytosed, eventually leading to down-regulation of receptor signalling. By blocking endocytosis, activated Rho and Rac (and possibly Cdc42) would prolong receptor signalling, leading to constitutive activation of downstream pathways. Some of these pathways may be down-regulated by feedback mechanisms.

acids — residues equivalent to 12, 59 and 61 of Ras — which are almost universally conserved in the Ras and Rho subfamilies are substituted with amino acids which make Ras oncogenic; in TTF, amino acids equivalent to residues 12 and 61 in Ras are similarly substituted. When tagged RhoE protein is expressed and immunoprecipitated from cells, it is found exclusively bound to GTP, and *in vitro* it does not detectably hydrolyse GTP, consistent with the hypothesis that the amino-acid substitutions (with respect to Ras) inhibit GTPase activity [2]. Indeed, when these three amino acids are mutated to the residues found in other Rho family members, the protein is now able to hydrolyse GTP. If RhoE really binds only to GTP, does it no longer act as a regulatable switch, turned on by the exchange of GDP for GTP? Maybe GTP is simply acting as an essential co-factor to maintain protein structure, and RhoE activity is regulated in some other way, for example by phosphorylation (as has been suggested for RhoA) [71], by altered cellular localization, or by reversible binding to an inhibitory protein such as Rho-GDI [1]. Clearly, we still have a lot to discover about this fascinating family of proteins.

Conclusions and perspectives

It is becoming increasingly clear that proteins of the Rho family carry out diverse functions and have the potential to interact with a large number of other proteins. The ability of GTP-binding proteins to communicate with many downstream targets is best illustrated by Ras itself, although the functional consequences of most of its interactions are still unclear. Studies using a panel of Ras mutants are beginning to provide information on the specific signalling roles of some Ras target proteins [72,73]; no doubt similar studies with the Rho family will help to determine which targets mediate the various responses they generate. In addition, expression of each target protein (Table 2) in cells, along with the isolation of interacting proteins, will provide important information on their downstream connections.

One of the major challenges in the Rho field is that of specificity. Not only are there 11 members of the Rho family, but there are ever-growing families of regulators and target proteins. One concern with many experimental approaches used to study protein function is that overexpression of a protein may artificially induce a response normally mediated *in vivo* by a related family member. In many cases, more detailed studies using a wide range of Rho family proteins and mutants will help to resolve this issue. In addition, investigating the intracellular localization of regulators, GTP-binding proteins and targets will elucidate which proteins are accessible to each other in cells.

In conclusion, it appears that members of the Ras superfamily have evolved to present a surface in their GTP-bound conformation capable of interacting in different ways with several proteins, and therefore coordinating the activation of multiple pathways. At the moment, we have many functions for Rho family proteins, and many targets, with only a few connections between the two. But research is moving so rapidly and productively in this field that no doubt many new links will be forged in the near future.

Acknowledgments

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