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Signal transduction through the GTP-binding proteins Rac and Rho

Anne J. Ridley

Ludwig Institute for Cancer Research, University College/Middlesex Hospital Branch, 91 Riding House Street, London W1P 8BT, UK

SUMMARY

Actin reorganization is an early response to many extracellular factors. In Swiss 3T3 fibroblasts, the Ras-related GTP-binding proteins Rho and Rac act as key signal transducers in these responses: Rho is required for growth factor-induced formation of stress fibres and focal adhesions, whereas membrane ruffling is regulated by Rac proteins. Several proteins that act as GTPase activating proteins (GAPs) for Rho-related proteins have been identified, and these could act either as targets or down-regulators of Rho or Rac in cells. In vitro, the GAP domain of p190 has a striking preference for Rho as a substrate, and when microinjected into Swiss 3T3 cells it inhibits stress fibre formation but not membrane ruffling induced by growth factors. BcrGAP acts on Rac but not Rho in vitro, and specifically inhibits membrane ruffling in vivo. Finally, RhoGAP acts preferentially on the Rho-related protein G25K/Cdc42Hs in vitro, but can inhibit Rho-mediated responses in vivo. These results suggest that p190, Bcr and RhoGAP play specific roles in signalling pathways through different Rho family members.

The mechanisms underlying Rho-regulated stress fibre formation have been investigated further by analysing the role of other signals known to be activated by lysophosphatidic acid (LPA). Neither activation of PK-C, increased intracellular Ca^{2+}, decreased cAMP levels or Ras activation appear to mediate stress fibre formation. However, LPA stimulates tyrosine phosphorylation of a number of proteins, including the focal adhesion kinase, $pp125^{FAK}$, and genistein, a tyrosine kinase inhibitor, prevents this increase in tyrosine phosphorylation. Genistein also inhibits LPA- and Rho-induced stress fibre formation, implying that a tyrosine kinase lies downstream of Rho in this signal transduction pathway.

Key words: Rho, Rac, GTPase, actin cytoskeleton, lysophosphatidic acid

INTRODUCTION

Rapid reorganization of the actin cytoskeleton is observed in cells in response to many extracellular factors. In fibroblasts, changes in two types of structure containing actin filaments, membrane ruffles and stress fibres, have been monitored following addition of growth factors (Ridley and Hall, 1992). Membrane ruffles and lamellipodia are plasma membrane protrusions that contain a dense network of cross-linked actin filaments, while stress fibres are bundles of actin filaments containing several other proteins including myosin, tropomyosin and α-actinin (Matsudaira, 1991; Stossel, 1993). At least one end of each stress fibre terminates at the plasma membrane in a focal adhesion, where integrins are clustered and link the extracellular matrix to the actin cytoskeleton (Burridge et al., 1988).

There is increasing evidence that a number of small GTP-binding proteins are involved in signal transduction pathways at the plasma membrane. The prototype for this is Ras, which has been shown to be involved in regulating responses leading to proliferation or differentiation (Downward, 1990). More recently, it has been shown that two related proteins, Rac and Rho, are required for growth factor-induced actin reorganization, at least in fibroblasts (Ridley and Hall, 1992; Ridley et al., 1992). It was found that microinjection of activated Rac induced the formation of membrane ruffles, resembling the response of cells to growth factors such as PDGF and EGF. Rho, on the other hand, stimulated the formation of stress fibres and focal adhesions, resembling the response to lysophosphatidic acid (LPA), a component of serum. By inhibiting the function of endogenous Rho or Rac proteins, it was shown that Rac is required for growth factor-induced membrane ruffling, whereas Rho is required for stress fibre formation. Inhibition of Rho was achieved by microinjecting C3 transferase, an exoenzyme produced by *Clostridium botulinum* that ADP-ribosylates and inactivates Rho proteins. Rac was inhibited by microinjecting the dominant inhibitory protein N17 Rac1, which has amino acid 17 mutated from threonine to asparagine.

Interestingly, although activated Ras induces membrane ruffling in fibroblasts, normal Ras is not required for growth factor-induced actin reorganization in Swiss 3T3 cells (Ridley et al., 1992). In addition, Ras-induced membrane ruffling was found to be dependent on Rac activity. This suggests that in these cells at least one response to tyrosine kinase receptors, actin reorganization, is not dependent on Ras, and that receptors feed into multiple small GTP-binding proteins to induce different responses.

Two closely related *Rac* genes, *Rac*1 and *Rac*2, have been cloned, and three *Rho* genes, *Rho*A, *Rho*B and *Rho*C. Other Rho-related proteins in mammalian cells include G25K and its close homologue Cdc42Hs, RhoG and TC10 (Nobes and Hall, 1994). The three Rho proteins are ~80% homologous, and only differ signficantly from each other at the C terminus; whether

these differences are functionally relevant is not clear. It is known, however, that the RhoB C-terminal sequences serve to target the protein predominantly to endosomes and lysosomes, whereas RhoA and RhoC C-terminal sequences target them to the plasma membrane and cytosol (Adamson et al., 1992).

Two lines of investigation have yielded further insight into the signal transduction pathways linking receptor activation to actin reorganization. First, the role of proteins, initially identified through their interaction with Rho or Rac in vitro, in regulating Rac- or Rho-dependent signalling in vivo has been studied. Second, the involvement of known signalling molecules or second messengers in regulating Rho- or Rac-mediated actin reorganization has been investigated.

PROTEINS INTERACTING WITH RHO AND RAC

Small GTP-binding proteins cycle between an active, GTP-bound form and an inactive, GDP-bound form. Extracellular signals are presumed to induce an increase in the amount of GTP-bound protein, and three mechanisms have been identified that could regulate this process: sequestration of the protein in an inactive complex with a GDI (guanine nucleotide dissociation inhibitor); stimulation of nucleotide exchange; and stimulation of GTP hydrolysis.

Little is known about the regulation of nucleotide exchange on Rac and Rho. The oncogene product Dbl has been shown to stimulate exchange on the related protein CDC42Hs, but is much less active on Rac (Hart et al., 1991). Several proteins with Dbl homology domains have been identified (Table 1; Nobes and Hall, 1994). As yet none of these proteins has been shown to have exchange factor activity on any member of the Rho family, although Ect2 has been shown to bind to Rac, Rho and Cdc42Hs (Miki et al., 1993). It is therefore possible that this domain represents a Rac/Rho interaction domain, and that only in the case of Dbl does this result in nucleotide exchange.

RhoGDI interacts with Rac, Rho and Cdc42Hs, inhibiting nucleotide exchange, GTP hydrolysis and interaction with GAPs (Nobes and Hall, 1994). In addition, it solubilizes the proteins from membranes, presumably by interacting with the hydrophobic prenyl groups attached to the C terminus of all Ras-related proteins (Isomura et al., 1991; Leonard et al., 1993). RhoGDI is therefore believed to sequester the proteins in an inactive, cytosolic form, and indeed in resting neutrophils it has been shown that Rac is predominantly cytosolic and bound to RhoGDI (Segal and Abo, 1993). Consistent with this model, microinjection of RhoGDI can inhibit Rho-dependent responses such as the formation of stress fibres (Miura et al., 1993).

Several proteins containing domains with GAP activity for Rho-related proteins have been identified and characterized, including RhoGAP, Bcr and p190 (Table 1; Hall, 1992; Boguski and McCormick, 1993). To determine whether these proteins exhibited any specificity in their GAP activity for different members of the Rho family, the GAP domains of each protein were tested for their relative GAP activities in vitro and following microinjection into Swiss 3T3 cells (Ridley et al., 1993). In vitro, at physiological ionic strength, RhoGAP was most effective as a GAP for G25K, while p190 acted on Rho, and Bcr acted on both Rac and G25K but not Rho (Table 2). When the domain of p190 was injected into Swiss 3T3 cells, it caused the cells to round up in a similar manner to C3 transferase. In addition, it inhibited LPA- and serum-induced stress fibre formation but not PDGF-induced membrane ruffling (Table 2). These results suggest that in cells as well as in vitro p190 acts primarily as a GAP for Rho and not for Rac. In contrast, the GAP domain of Bcr inhibited PDGF-induced membrane ruffling but not LPA-induced stress fibre formation, indicating that Bcr acts as a GAP for Rac but not Rho in cells. RhoGAP, which acts primarily on G25K in vitro, inhibited stress fibre formation when microinjected into cells, but did not inhibit membrane ruffling. An action of growth factors dependent on G25K/Cdc42Hs has so far not been described, so it was not possible to analyse the ability of these GAPs to down-regulate G25K.

These results suggest that each GAP acts specifically in cells to downregulate either Rac or Rho, and therefore that the proteins are likely to act as components of one or other signal transduction pathway leading either to membrane ruffling or stress fibre formation. Whether they act merely as downregulators of Rac or Rho activity, or whether their GAP activity is normally linked in cells to their activation by Rho or Rac, is not known. It is interesting in this respect that both Bcr and p190 have other distinct domains with putative or known functions (Fig. 1). For example, Bcr has a domain with serine/threonine kinase activity (Maru and Witte, 1991), while

Table 1. Potential regulators of Rho-related proteins

(A) *Proteins with Dbl-homologous domains*	
Dbl	Exchange factor activity for CDC42Hs
Bcr	Dbl domain in Bcr-Abl in some leukemias
Vav	Oncogene
Ect2	Oncogene
Sos	Exchange factor for Ras
RasGRF	Exchange factor for Ras
(B) *Guanine nucleotide dissociation inhibitors (GDIs)*	
RhoGDI	Ubiquitous expression?
LyGDI	Lymphocytes
D4	Haemopoietic cells
(C) *Proteins with GAP domains*	
RhoGAP	Active on Rho and G25K/Cdc42Hs
p190	Binds RasGAP
Bcr	Bcr-Abl fusion protein in leukemias
3BP-1	Binds SH3 domains
n-chimaerin	Brain-specific expression
β-chimaerin	Testis-specific expression
p85	Subunit of PI 3-kinase; no GAP activity shown

Table 2. Specificities of GAPs for Rac, Rho and G25K

GAP domain from:	In vitro GAP activity	Microinjected GAP domains
p190	Rho	Rho
Bcr	Rac/G25K	Rac
RhoGAP	G25K	Rho

The best substrate for each GAP domain was determined in vitro by testing their relative GAP activities on RhoA, Rac1 and G25K. In cells, the preference for Rho or Rac was determined by microinjecting each domain, and testing for its ability to inhibit either stress fibre formation or membrane ruffling (Ridley et al., 1993). It was not possible to determine their ability to act as GAPs for G25K in cells, as no measurable biological function of G25K has been described.

Fig. 1. Domain structures of GTPase activating proteins for Rac and Rho. Diagrammatic representations of the proteins RhoGAP, p190 and Bcr are shown to illustrate the relative positions of different domains; they are not to scale. See text for references.

p190 has a domain homologous to GTP-binding proteins and may therefore itself bind GTP (Settleman et al., 1992). It remains to be determined whether other members of the RhoGAP family also show activity on Rho or Rac in cells, and whether the GAPs tested so far act on other Rho-related proteins such as RhoG and TC10.

The best-characterized system so far involving a Rho-related protein is the NADPH oxidase of phagocytic cells, where Rac1 or Rac2 is required for activation of the complex at the plasma membrane (Segal and Abo, 1993). All of the components of this complex have been cloned, and NADPH oxidase activity can be reconstituted in vitro. This action of Rac is quite distinct from its ability to stimulate membrane ruffling, as the other components of the complex are not expressed in fibroblasts (Morel et al., 1991).

Recently, a serine/threonine kinase, $p65^{PAK}$, was identified by its ability to interact in vitro with Rac and Cdc42Hs bound to GTP. It was purified from brain extracts, leading to amino acid sequence and cDNA cloning (Manser et al., 1993). The kinase activity of $p65^{PAK}$ is stimulated by Rac and Cdc42Hs, and it may therefore be a Rac/Cdc42Hs target, although at least in vitro it is activated by both proteins and therefore not a specific target.

SIGNAL TRANSDUCTION PATHWAYS REGULATING RHO-MEDIATED STRESS FIBRE FORMATION

LPA is the only extracellular factor tested so far that induces only stress fibre formation and not membrane ruffling in Swiss 3T3 cells. In addition, the second messengers generated in response to LPA have been characterized in detail in fibroblast cell lines (Moolenaar et al., 1992). The LPA response is therefore well-suited to analysis of the signal transduction pathways regulating stress fibre formation.

LPA is the simplest naturally occuring phospholipid, consisting of a glycerol backbone with a fatty acid esterified at position 1 and a phosphate at position 3. It appears to act primarily through a transmembrane receptor, and a putative receptor of approximately 39 kDa has been identified but not yet cloned (van der Bend et al., 1992). LPA is produced by activated platelets and is therefore present in serum, and several biological activities for LPA have been characterized (Table 3).

Early signals activated or generated by LPA in fibroblasts and other cell types are illustrated in Fig. 2. First, LPA activates a Pertussis toxin-sensitive G protein, leading to a decrease in intracellular cAMP levels and activation of Ras.

Table 3. Biological actions of LPA

Cell type	Activity
Fibroblast	DNA synthesis
	Stress fibre formation
	Focal adhesion formation
Platelet	Aggregation
Neuron	Growth cone retraction
Smooth muscle	Contraction
Dictyostelium	Chemotaxis

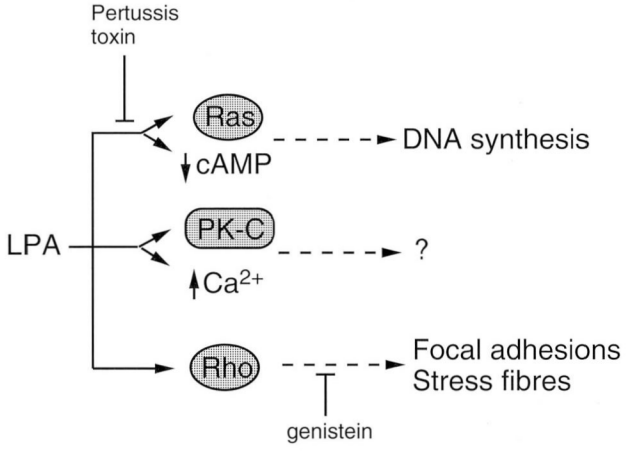

Fig. 2. Signalling pathways activated by LPA. LPA activates at least three distinct signalling pathways in fibroblasts. First, it activates a G_i protein, leading to a decrease in cAMP levels, activation of Ras and downstream signals culminating in DNA synthesis. Second, it activates a phospholipase C, leading to stimulation of protein kinase C isoforms and release of Ca^{2+} from intracellular stores. Third, it activates a Rho-dependent pathway involving tyrosine phosphorylation of proteins, and leading to the formation of focal adhesions and stress fibres.

Neither of these signals were required for stress fibre formation, as the latter was not sensitive to Pertussis toxin (Ridley and Hall, 1994). In addition, neither increasing cAMP levels nor microinjection of neutralizing anti-Ras antibodies inhibited LPA-induced stress fibre formation (Ridley et al., 1992; Ridley and Hall, 1994). Second, LPA stimulates a phospholipase C leading to production of the second messengers inositol trisphosphate (IP_3) and diacylglycerol; the former stimulates release of Ca^{2+} from intracellular stores, whereas the latter activates protein kinase C (Moolenaar et al., 1992). Mimicking either or both of these responses did not stimulate stress fibre accumulation: the Ca^{2+} ionophore A23187 or the PK-C-activating phorbol ester PMA were used to mimic these signals. Third, LPA activates another, as yet undefined pathway, which is dependent on protein kinase activation (Jalink et al., 1993), and indeed LPA was found to stimulate the tyrosine phosphorylation of a number of proteins (Kumagai et al., 1993; Ridley and Hall, 1994). As mentioned above, LPA rapidly induces the formation of focal adhesions as well as stress fibres, and it has been shown previously that phosphotyrosine-containing proteins are located at focal adhesions in growing cells, using anti-phosphotyrosine antibodies (Maher et al., 1985; Burridge et al., 1992). We showed that LPA stimulated the accumulation of phosphotyrosine-containing proteins in focal adhesions very rapidly, within one minute of

addition (Ridley and Hall, 1994). This process was dependent on endogenous Rho proteins as it was inhibited in cells microinjected with C3 transferase. Microinjected activated V14 RhoA similarly stimulated the clustering of phosphotyrosine-containing proteins at the ends of newly formed stress fibres.

To test whether the formation of stress fibres was dependent on tyrosine kinase activity, we used the kinase inhibitor genistein. This has been shown to inhibit several tyrosine kinases in vivo, and to act preferentially on tyrosine kinases compared to serine/threonine kinases such as protein kinase C or protein kinase A (Akiyama et al., 1987). Genistein inhibited both LPA- and Rho-induced stress fibre formation, and LPA-induced clustering of phosphotyrosine-containing proteins in focal adhesions. Genistein was clearly acting as a tyrosine kinase inhibitor in these cells, as at the same concentration of genistein, the increased tyrosine phosphorylation of several proteins induced by LPA and by bombesin was inhibited. Specifically, increased phosphorylation of pp125^{FAK} (focal adhesion kinase) was inhibited. pp125^{FAK} is a tyrosine kinase located in focal adhesions, which is activated by tyrosine phosphorylation following adhesion of fibroblasts to extracellular matrix proteins such as fibronectin (Schaller and Parsons, 1993). Interestingly, tyrosine phosphorylation of pp125^{FAK} is also stimulated by bombesin (Zachary et al., 1992), which induces stress fibre formation with a similar timecourse to LPA. Our data suggest that a tyrosine kinase acts downstream of Rho and is required for focal adhesion and stress fibre formation, although we cannot formally rule out the possibility that the primary target for genistein in this process is not a tyrosine kinase. Whether pp125^{FAK} plays a regulatory role in the formation of focal adhesions and/or stress fibres remains to be determined.

CONCLUSIONS

It has long been known that Ras is involved in early signal transduction events induced by a variety of extracellular factors. Over the past two years it has become clear that Ras-related proteins of the Rho subfamily are also involved in signal transduction. The plethora of signal transduction proteins and/or oncogene products recently shown to interact with Rho-related proteins indicates a fundamental role for these proteins in cellular responses. The available data suggest that in fibroblasts Rho is most likely to be involved in regulating cell adhesion to the extracellular matrix, and that stress fibres are formed as a consequence of this. In T cells it has been shown that C3 transferase inhibits integrin-mediated cell-cell interactions (Tominaga et al., 1993), suggesting that a more general role of Rho in various cell types could be to regulate integrin-mediated interactions. Rac, on the other hand, in regulating membrane ruffling and lamellipodia extension, is likely to be involved in cell motility responses.

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