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THE ROLE OF RHO IN G PROTEIN-COUPLED RECEPTOR SIGNAL TRANSDUCTION

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■ **Abstract** Low molecular weight G proteins of the Rho subfamily are regulators of actin cytoskeletal organization. In contrast to the heterotrimeric G proteins, the small GTPases are not directly activated through ligand binding to G protein–coupled receptors (GPCRs). However, a subset of GPCRs, including those for lysophosphatidic acid and thrombin, induce stress fibers, focal adhesions, and cell rounding through Rho-dependent pathways. C3 exoenzyme has been a useful tool for demonstrating Rho involvement in these and other responses, including Ca^{2+} sensitization of smooth muscle contraction, cell migration, transformation, and serum response element–mediated gene expression. Most of the GPCRs that induce Rho-dependent responses can activate G_q, but this is not a sufficient signal. Recent data demonstrate that $G\alpha_{12/13}$ can induce Rho-dependent responses. Furthermore, $G\alpha_{12/13}$ can bind and activate Rho-specific guanine nucleotide exchange factors, providing a mechanism by which GPCRs that couple to $G\alpha_{12/13}$ could activate Rho and its downstream responses.

INTRODUCTION

Low molecular weight G proteins are well recognized as mediators of cell growth and actin cytoskeletal rearrangement in mammalian cells. The discovery that extracellular stimuli regulate these proteins suggested their role in signal transduction pathways. Studies in the early 1990s focused on the Ras family proteins and delineated the steps leading to Ras and mitogen-activated protein (MAP) kinase activation by receptor tyrosine kinases. G protein–coupled receptors (GPCRs) were also demonstrated to activate Ras and MAP kinase cascades, albeit with relatively low efficacy compared with receptor tyrosine kinases. Although interest in small G proteins of the Rho family was limited, a role for Rho in signal transduction had also been discovered in the early 1990s. This was demonstrated not through the effects of tyrosine kinase growth factors, but rather through the remarkable effects of the GPCR agonists lysophosphatidic acid (LPA), thrombin, bombesin, and endothelin on cell morphology and tyrosine phosphorylation. The

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concept that Rho proteins are mediators of responses to certain GPCRs has now become well recognized and is the subject of this and a previous review (1).

PROPERTIES AND REGULATORS OF RHO FAMILY PROTEINS

Rho Family Proteins

The first Rho family protein was identified as a Ras homolog in the sea snail *Aplysia* (2). Currently at least 14 distinct Rho family proteins ranging from 20–25 kDa have been identified. These can be broadly divided into subfamilies (Rac, Cdc42, Rnd, and Rho) based on amino acid sequence identities and cellular functions (reviewed in 3, 4). The members of the Rho subfamily, RhoA, RhoB, and RhoC, share >85% homology. Differences in their lipid modification have been proposed to influence their interaction with regulators and their subcellular localization (5). RhoA is the best-characterized member of the Rho family of low molecular weight GTPases. Most studies examining cellular responses to Rho have utilized transiently expressed or microinjected RhoA protein or cDNA expression plasmid. On the other hand, studies implicating Rho in cellular responses through the use of inhibitors such as C3 exoenzyme, dominant negative Rho, guanine nucleotide dissociation inhibitors (GDIs), or mutant guanine nucleotide exchange factors (GEFs) do not target specific Rho subfamily members (RhoA vs RhoB vs RhoC). Thus, although it is assumed that it is RhoA that regulates the pathways discussed in this review, the more general terminology Rho is used.

Studies investigating the involvement of Rho in various cellular responses have been facilitated by the generation of mutant proteins that interfere with or enhance Rho function. G proteins cycle between an inactive GDP-bound state and an active GTP-bound state. Studies of Ras identified several critical amino acids that modulate the GTP-bound state. Analogous mutations were made in RhoA. Mutation of Ser^{19} to Asn^{19} results in a protein with increased affinity for GEFs. Hence, by competing for required activators, these proteins serve as dominant negative inhibitors of endogenous Rho activation. Dominant negative Rho proteins have been widely used to block Rho-dependent responses (see, for example, 6–8) but have been noted to be unstable (P Chardin, personal communication). Conversely, mutation of Gly^{14} to Val^{14} or of Gln^{63} to Leu^{63} on RhoA renders the protein GTPase deficient and thus constitutively GTP-bound and active.

RhoGAPs, GEFs, and GDIs

In vivo, the activation of Rho is regulated by GTPase activating proteins (GAPs), GEFs, and GDIs and can be modulated by bacterial toxins. GAPs regulate the inactivation of small G proteins by accelerating their intrinsic GTPase activity.

Protein tyrosine phosphatase PTPL1-associated RhoGAP (PARG1) has been shown to have GAP activity for Rho family GTPases, with a preference for Rho (10). Other Rho-specific GAPs include Graf (11, 12), p190RhoGAP (13), and p122RhoGAP (15). Graf and p190RhoGAP have been demonstrated to localize to the actin cytoskeleton, providing a possible mechanism for rapid termination of Rho-mediated cytoskeletal rearrangements. Graf is ubiquitously expressed and can be phosphorylated by MAP kinase (11). The p190RhoGAP is tyrosine phosphorylated in response to activation of c-Src, resulting in enhanced RhoGAP activity and actin disorganization (13, 14). The p122RhoGAP, when transiently transfected or microinjected into Swiss 3T3 cells, inhibits LPA-stimulated, Rho-dependent stress fiber formation (15). The concept that the activity and localization of GAPs may be regulated makes them potential targets in the control of responses to GPCRs.

RhoGEFs catalyze the exchange of GDP for GTP and thereby activate Rho. The proteins in this family contain a number of well-characterized domains. The dbl homology (DH) domain, named for Dbl the first identified Rho family GEF (16), and an adjacent pleckstrin homology (PH) domain are common to all Rho GEFs. The DH domain possesses the nucleotide exchange activity, and the PH domain contributes to this as well as to the cellular localization of the GEF. Microinjection or expression of RhoGEFs has been shown to induce changes in cell shape, gene expression, DNA synthesis, and cell transformation (17–20). Conversely, mutant forms of RhoGEFs lacking DH domains have been used as inhibitors of GPCR responses requiring Rho function (17–19).

Many of the RhoGEFs were first identified as oncogenes in DNA isolated from malignant cells. Dbl stimulates guanine nucleotide exchange on both Cdc42 and RhoA (16). Other Rho-specific GEFs isolated as oncogenes include Lbc (20), Lfc (21), and Lsc (22). RIP2 is a putative RhoGEF isolated as a Rho-interacting protein (RIP) using the yeast two-hybrid system (23). In addition to DH and PH domains, RIP2 contains a leucine-rich motif and a zinc-finger–like motif similar to those present in Lfc. These domains could contribute to protein or DNA binding. The p115RhoGEF (24) also contains regulatory sites in addition to DH and PH domains. This GEF has an N-terminal domain similar to that found in regulators of G protein signaling (RGS) proteins and has been shown to catalyze GTPase-stimulated inactivation of $G\alpha_{12/13}$ proteins (25). Another GEF was named PDZ-RhoGEF based on inclusion of an N-terminal PDZ domain (17). It is the lsc homology (LH) domain that is of greater potential interest for GPCR signaling, however, because this domain (which shows limited sequence similarity to RGS14) is required for binding of PDZ-RhoGEF to $G\alpha_{12}$ and $G\alpha_{13}$. The RGS and related LH domains in RhoGEFs may provide negative feedback at the level of $G\alpha$ subunits for GPCR-mediated responses. Another group of RhoGEFs, including Trio (26) and Duet (27), possess serine/threonine kinase activity. Thus, RhoGEFs may regulate responses other than the activation of small G proteins.

GDIs bind Rho family GTPases, targeting the major fraction of Rho to the cytosol in unstimulated cells (for reviews, see 28, 29). Binding of Rho to

RhoGDIs inhibits guanine nucleotide exchange and activation of the Rho GTPases. The ubiquitously expressed RhoGDI as well as GDI/D4, expressed only in hematopoietic cells, inhibit nucleotide exchange on all the Rho family proteins (RhoA, Rac and Cdc42). On the other hand, the homologous protein RhoGDIγ, which is preferentially expressed in brain and pancreas, only binds Rho and Cdc42 (29a). The RhoGDIs have been shown to inhibit various Rho-dependent functions, such as cell spreading and stress fiber formation in baby hamster kidney cells (29a), exocytosis in mast cells (30), and activation of phospholipase D (PLD) in response to GTPγS in plasma membranes from rat liver (31). Although GDIs show little relative specificity for particular Rho family members, they possess several protein domains subject to modification by serine-threonine kinases, which suggests their potential for regulation (for a review, see 29).

Bacterial Toxins

A number of bacterial toxins that inactivate Rho have been identified (reviewed in 32). The clostridial cytotoxins *Clostridium difficile* toxin A and toxin B inactivate all Rho family proteins by glucosylating the nucleotide binding site. Another family of Rho-inactivating enzymes consists of ADP-ribosyltransferases, including the *Clostridium botulinum* C3 exoenzyme, the *Clostridium limosum* transferase, and the *Staphylococcus aureus* transferase epidermal differentiation inhibitor. These toxins show greater specificity than those of the *C. difficile* family for Rho subfamily proteins (RhoA, RhoB, and RhoC). Although Rac was originally identified as a substrate for C3 exoenzyme (33), it has been demonstrated that C3-catalyzed ribosylation of RhoA is at least 100–400 times more efficient than that of Rac or Cdc42 (34). Thus, C3 can specifically target the Rho subfamily although they do not distinguish between RhoA, RhoB, or RhoC.

C3 exoenzyme irreversibly ADP-ribosylates Rho at Asn^{41} located in the effector region. Mutation of this residue to Ile^{41} prevents ribosylation by C3 (35). The exact mechanism by which ADP-ribosylation confers loss of Rho function and inhibition of Rho-mediated responses is unclear. One hypothesis is that ribosylation renders Rho unstable. Indeed, several studies have noted a substantial loss of Rho protein following C3 treatment (36, 37, 37a). Another possibility is that ribosylation alters RhoA localization. This is supported by a recent study demonstrating that GTPγS treatment increases RhoA localization in caveolar membranes, and that C3 pretreatment leads to a loss of RhoA from this compartment (38). Likewise, localization of RhoA in caveolae has been associated with cytoskeletal reorganization in astrocytes stimulated with endothelin-1 (38a).

The purified C3 exoenzyme possesses no cell surface binding or translocation components, thus various modes of achieving C3 expression and Rho blockade in intact cells have been devised. One strategy is to express the C3 cDNA by plasmid transfection (39) or Sindbis viral infection (37). Alternatively, purified recombinant C3 exoenzyme has been introduced into the cells by scrape-loading

(36), permeabilization (40), osmotic shock (41), or electroporation (42). A hybrid toxin consisting of the cell-binding and translocation subunit of the diphtheria toxin fused to C3 exoenzyme has also been used (43). The most common approaches have been either microinjection or prolonged incubation of cells with purified C3, which results in passive uptake (see, for example, 34, 35, 77, 83).

Measurement of Rho Activation

There is a substantial body of work utilizing tools such as C3 exoenzyme and dominant negative Rho to support the involvement of Rho in GPCR-mediated responses. In contrast, relatively few studies have directly examined the ability of GPCRs to activate Rho. As previously mentioned, the major cellular fraction of Rho is cytosolic, and upon stimulation by either guanine nucleotide or GPCR agonist, the amount of Rho associated with the membrane fraction is increased while cytosolic Rho is decreased. This phenomenon has been exploited in order to measure activation of Rho in response to LPA or endothelin in Swiss 3T3 fibroblasts (44), thrombin in astrocytoma and vascular smooth muscle cells (45, 46), and angiotensin II in cardiac myocytes (47). Increases in membrane-associated Rho have also been detected in response to GTPγS or GTP plus phenylephrine in permeabilized blood vessels (48), and decreases in cytosolic Rho have been described following addition of carbachol to permeabilized GTPγS-stimulated HEK cells (49).

Activation of Rho based on increased GTP binding has been more difficult to demonstrate, but a few groups have reported increased binding of radiolabeled guanine nucleotides to Rho following GPCR stimulation. Formylmethionylleucylphenylalanine (fMLP) increased both [^{32}P]GTP- and [^{32}P]GDP-bound Rho in leukocytes (50, 51). [^{35}S]GTPγS binding to Rho was increased in response to fMLP in leukocytes and in response to thrombin in rat aortic smooth muscle cells (46, 51). α_2-Adrenergic receptor stimulation increased [^{32}P]GTP binding to Rho and decreased [^{32}P]GDP-Rho binding in preadipocytes (52). Expression of various constitutively activated Gα subunits of heterotrimeric G proteins in COS-7 cells has also been shown to increase Rho-[^{32}P]GTP binding (53).

Recent studies have used Rho binding proteins in pull-down assays to measure Rho activation (54, 55). This method is based on the enhanced ability of activated (GTP bound vs GDP bound) Rho to bind the Rho binding domain (RBD) of Rho effectors. GST-fusion proteins of the RBDs of Rho kinase (54) and rhotekin (55) have been generated and used to affinity precipitate activated Rho. Using this assay, stimulation of COS-7 or Swiss 3T3 cells with LPA was shown to increase the amount of activated Rho (54, 55). Expression of activated $G\alpha_{12}$ or $G\alpha_{13}$ in COS-7 cells also resulted in increased Rho-RBD binding (54). The development of these apparently more sensitive Rho-RBD assays should expedite the elucidation of the molecular mechanisms involved in Rho activation in response to GPCR stimulation.

RHO-MEDIATED CELLULAR RESPONSES

Effectors of Rho

A large number of Rho-binding proteins have been identified by gel overlay, yeast two-hybrid screening, and related approaches (reviewed in 56, 57). Among the many Rho effectors identified, the serine/threonine-directed Rho kinases (Rho kinase/ROKα/ROCK-II and p160ROCK/ROCKβ, hereafter generically referred to as Rho kinase) are the best characterized (58–61).

Dominant negative mutants of Rho kinase have been utilized to demonstrate a requirement for Rho kinase in various cellular responses (see, for example, 58, 62–64, 98). Studies assessing the involvement of Rho kinase in cellular responses have been facilitated by the development of a selective inhibitor, Y27632 (65). Y27632 acts as a competitive inhibitor of ATP binding and has been shown to be ~200 times more selective for inhibiting Rho kinase than protein kinase C (PKC), cAMP-dependent protein kinase, and myosin light chain (MLC) kinase (65). In addition, effector mutants of RhoA that are unable to interact with Rho kinase have been generated (66, 67). Experiments using the aforementioned tools confirm a requirement for Rho kinase in the regulation of stress fibers, focal adhesions and cell transformation.

Much less is known about the specific functions of another group of Rho effectors that are PKC–related serine/threonine kinases (PKN/PRK1 and PRK2) (68–70). PKN has been demonstrated to be phosphorylated in a C3-sensitive manner on stimulation of Swiss 3T3 cells with LPA (69). PRK2 has been shown to cooperate with RhoA to induce serum response factor (SRF)-dependent transcriptional activation (70). However, studies with RhoA effector domain mutants indicate that RhoA-mediated stress fiber formation, SRF activation, and transformation can occur in the absence of RhoA-PKN interactions (66). Other Rho effectors include citron kinase, which regulates cytokinesis (71), p140mDia, which regulates actin reorganization (72), and rhophilin and rhotekin (68, 73), the functions of which remain unknown.

Cytoskeletal Responses

Pioneering work by several independent laboratories in the early 1990s established a direct role for Rho in the regulation of the actin cytoskeleton. Elegant microinjection experiments (34, 35) demonstrated that a constitutively active RhoA mutant stimulated actin stress fiber formation and focal adhesion complex assembly in serum-starved Swiss 3T3 cells, whereas inactivation of Rho prevented these serum-induced cytoskeletal responses. The factor in serum that was responsible for these cytoskeletal effects was later identified as LPA (34), an agonist that is now known to act through a GPCR (74). Other GPCR agonists, including endothelin and bombesin, were subsequently shown to elicit stress fiber formation and focal adhesion complex assembly in a Rho-dependent manner (75,

76). A distinct but related Rho-dependent cytoskeletal response characterized by process retraction and cell rounding is observed in neuronal and astroglial cells stimulated by GPCR agonists such as LPA and thrombin (77–79).

Studies by Rozengurt and coworkers (80–82) demonstrated that stimulation of GPCRs also led to rapid tyrosine phosphorylation of the cytoskeleton-associated proteins, p125 focal adhesion kinase (FAK) and paxillin, and to their clustering at focal adhesions. Agonist- or GTPγS-induced tyrosine phosphorylation of these proteins was not dependent on PKC activation or Ca^{2+} mobilization but was inhibited by C3 exoenzyme (75, 80–84), implicating Rho as a mediator of these responses. It is interesting to note that cytochalasin D, which disrupts the actin filament network, prevented tyrosine phosphorylation of FAK and paxillin in response to GPCR activation. This finding suggests that the response is dependent on the integrity of the actin cytoskeleton (80–82). Tyrosine phosphorylation of FAK and paxillin results in the creation of binding sites for other proteins, e.g. Src family kinases and phosphatidylinositol 3-kinase (PI(3)K), facilitating their recruitment to focal adhesion complexes for structural or signaling functions (85, 86). However, it is not yet clear how the phosphorylation of FAK and paxillin and recruitment of structural and signaling molecules to focal adhesion plaques contribute to downstream Rho-dependent responses.

The basis for the involvement of the actin cytoskeleton in focal adhesion formation and associated tyrosine phosphorylation is suggested by the work of Chrzanowska-Wodnicka & Burridge (87). They demonstrated that stimulation of fibroblasts with LPA increased MLC phosphorylation with a time course preceding that for the detection of tyrosine phosphorylation, stress fibers, and focal adhesions. Pharmacological inhibition of MLC kinase activity with KT5926 prevented the formation of stress fibers and focal adhesions (87), which suggests a role for contractile responses mediated through MLC phosphorylation.

Recent studies have shown that Rho and Rho kinase regulate MLC phosphorylation. Rho kinase phosphorylates the myosin-binding subunit of MLC phosphatase, rendering the phosphatase inactive and thus preventing MLC dephosphorylation (88, 89). In addition, Rho kinase has been reported to directly phosphorylate MLC in vitro (89). Together, these events result in an accumulation of phosphorylated MLC that promotes actin-myosin interaction. Considerable evidence indicates that Rho kinase mediates LPA-, thrombin-, and RhoA-induced actin stress fibers, focal adhesion complexes, endothelial cell contractility, and cell rounding through its effect on MLC phosphorylation (63, 78, 90–93). Thrombin-induced cell rounding and MLC phosphorylation in 1321N1 astrocytoma cells are C3-sensitive and prevented by Y27632 (19, 78). In N1E-115 cells, expression of activated Rho kinase was shown to promote MLC phosphorylation and neurite retraction, and dominant negative Rho kinase blocked both of these responses. In addition, a mutant MLC (T18D, S19D) that mimics the phosphorylated state of myosin also induced neurite retraction (91).

Although numerous studies suggest an association between MLC phosphorylation and cytoskeletal reorganization (42, 78, 87, 90–93), some caution in inter-

pretation is warranted. The MLC kinase inhibitor KT5926 was recently shown to inhibit PKC (42). Additionally, KT5926 completely inhibits astrocytoma cell rounding at concentrations that only partially blocked MLC phosphorylation (78). These observations suggest some dissociation between MLC phosphorylation and the cytoskeletal response. Other mechanisms by which Rho and Rho kinase could mediate actin cytoskeletal responses must therefore be considered. LPA has been demonstrated to phosphorylate ezrin/radixin/moesin (ERM) proteins in a Rho-dependent manner (94). Furthermore, phosphorylation of ERM proteins is regulated by Rho kinase and the myosin-binding subunit of myosin phosphatase (94, 95). ERM phosphorylation regulates the ability of these proteins to cross-link the plasma membrane and actin filaments. Additionally, Maekawa et al recently identified LIM kinase as a Rho kinase target involved in stress fiber formation in HeLa cells (96). These investigators reported that LIM kinase is phosphorylated and activated by Rho kinase, resulting in phosphorylation of cofilin, an actin depolymerizing protein. Phosphorylation of cofilin has been demonstrated to suppress its activity, thus contributing to actin cytoskeletal reorganization. Although neither LIM kinase nor ERM proteins has been demonstrated to be involved in GPCR signaling to the cytoskeleton, investigations into the role of these proteins are likely to be forthcoming.

Stress fiber formation in fibroblasts has also been suggested to occur via activation of the ubiquitously expressed Na^+-H^+ exchanger NHE1 (97), which regulates intracellular pH homeostasis and is associated with cellular growth responses. Barber's laboratory demonstrated that the induction of stress fibers by LPA and activated RhoA was abolished in NHE1-deficient cells and also by treatment with ethylisopropylamiloride, a pharmacological inhibitor of NHE1 (97). LPA-stimulated phosphorylation and activation of NHE1 in vivo was inhibited by a catalytically inactive Rho kinase or pretreatment of the cells with Y27632, which suggests that Rho kinase mediates LPA- and RhoA-induced NHE1 activity (98).

Phospholipid Metabolism

A number of phospholipid metabolizing enzymes appear to be regulated through Rho-dependent pathways. PI(3)K is an enzyme known to signal responses from receptor tyrosine kinases and to regulate the actin cytoskeleton (reviewed in 99). GPCRs including the thrombin receptor in platelets and the LPA receptor in Swiss 3T3 cells have been shown to activate PI(3)K, and studies using C3 exoenzyme indicate that this requires Rho function (83, 100). The isoform of PI(3)K regulated by Rho in platelets is the p85/p110 heterodimer. In contrast, the p110 catalytic subunit, PI(3)Kγ, which is regulated by GPCRs and controlled through G$\beta\gamma$ subunits, is not Rho-dependent (101). The products of PI(3)K, PI(3,4)P_2, and PI(3,4,5)P_3 function in the regulation of downstream effectors such as Akt/PKB (99), thus alterations in PI(3)K activity could contribute to Rho-mediated apoptosis (see below).

Phosphatidylinositol-4-phosphate 5-kinase (PIP5K), another Rho-activated phosphoinositide kinase, has been shown to interact with both GTP- and GDP-bound recombinant Rho (102). GTP-bound Rho and GTPγS increase PIP5K activity, and this is inhibited by C3 exoenzyme (103). PIP5K catalyzes the resynthesis of PI(4,5)P_2, the substrate for phospholipase C (PLC). Because cellular PI(4,5)P_2 levels are limited, PI(4,5)P_2 resynthesis is required to prevent depletion of hormonally regulated stores of this lipid. Accordingly, C3 treatment was shown to attenuate Ca^{2+} mobilization by PLC-coupled receptors (103). Similar conclusions were reached in studies examining PI(4,5)P_2 levels and inositol phosphate formation in N1E-115 cells (104). In these cells, inositol phosphate formation induced by bradykinin and LPA was inhibited by pretreatment with C3 exoenzyme and *C. difficile* toxin B. This was associated with a marked reduction in total cellular PI(4,5)P_2 levels in the absence of diminished PLC catalytic activity (104). Thus Rho-dependent pathways can regulate the supply of PI(4,5)P_2 needed to sustain Ca^{2+} mobilization and presumably PKC signaling by PLC-coupled receptors.

Changes in PI(4,5)P_2 levels could also contribute to control of the actin cytoskeleton. PI(4,5)P_2 associates with actin binding proteins such as profilin and gelsolin, uncaps the barbed ends of actin filaments, and promotes actin polymerization. Microinjection of PI(4,5)P_2 antibodies inhibits assembly of stress fibers and focal adhesions (105). Conversely, when PIP5K was microinjected into COS-7 cells, actin polymerization was induced (106). The delta isoform of PLC is also regulated by PI(4,5)P_2, and both Rho and p122RhoGAP have been suggested to directly modulate PLCδ_1 activity (15, 107).

Stimulation of the LPA, endothelin, m_3 muscarinic, bradykinin, sphingosine 1-phosphate, and α_2-adrenergic GPCRs leads to Rho-dependent PLD activation (36, 37, 40, 108, 109). A GTPase-deficient $G\alpha_{13}$ mutant also stimulated PLD activity in a C3-sensitive manner (110). The Rho-dependent stimulation of PLD by the m_3 mAChR was inhibited by Rho kinase mutants and a Rho kinase inhibitor, HA-1077, which suggests that this response is mediated through Rho kinase-dependent phosphorylation (111). How Rho functions in the regulation of PLD activity is still unclear. Although Rho may regulate PLD indirectly via its effect on synthesis of the PLD cofactor PI(4,5)P_2, several studies demonstrate a direct interaction between Rho and PLD (112, 113).

Smooth Muscle Contraction

The traditional Ca^{2+}-dependent biochemical pathway responsible for vascular smooth muscle contraction has been well characterized. Heterotrimeric G protein-linked contractile agonists that couple to G_q and/or G_i increase intracellular Ca^{2+}, and subsequently Ca^{2+}-bound calmodulin activates MLC kinase. Increases in the phosphorylation state of MLC stimulate the actinomyosin ATPase, resulting in cross bridge cycling and contraction. A mechanism for Rho involvement in GPCR stimulation of vascular contraction has been more recently elucidated. Initial stud-

ies revealed that in permeabilized blood vessels, where Ca^{2+} concentrations can be maintained constant, nonhydrolyzable GTP analogs or GTP plus agonists elicit a contractile response (114, 115). This led to the hypothesis that a G protein(s) is involved in Ca^{2+} sensitization, i.e. contraction in the absence of increases in intracellular Ca^{2+}. The observation that C3 exoenzyme blocked agonist and guanine nucleotide-induced contraction of permeabilized vessels (116, 117) and the associated increase in MLC phosphorylation (118) led to the conclusion that Rho is responsible for Ca^{2+} sensitization. Consistent with this theory, a study by the Somlyo laboratory showed that redistribution of Rho to the plasma membrane correlated with Ca^{2+} sensitization (48).

The mechanism for this Rho-dependent response has been elucidated. As described previously, activation of Rho kinase leads to accumulation of phosphorylated MLC (88). Addition of the catalytic subunit of Rho kinase to permeabilized vessels results in contraction (120) whereas Y27632 inhibits contraction induced by phenylephrine or GTPγS (65). These data provide evidence that Rho kinase is the effector that mediates Ca^{2+} sensitization. Recently, Rho kinase-mediated Ca^{2+} sensitization has been implicated in the pathophysiology of hypertension. Narumiya's laboratory has shown that acute administration of Y27632 reduces blood pressure in three forms of experimental hypertension (65). This observation along with the previously observed increase in serotonin-stimulated Ca^{2+} sensitization in permeabilized vessels from hypertensive rats suggests that Ca^{2+} sensitization may be enhanced in hypertension (121). Studies performed in our laboratory reveal a role for Rho and Rho kinase as mediators of thrombin-stimulated vascular smooth muscle cell DNA synthesis and migration (46), two responses thought to be enhanced in experimental hypertension and possibly involved in the pathophysiology of atherosclerosis, restenosis, and graft rejection.

Cell Migration and Tumor Cell Invasion

Cell migration is required for physiological processes such as embryonic development, wound healing, and inflammation as well as for pathophysiological responses such as atherosclerosis and metastasis of cancer cells. Recently, Rho has been established as a critical mediator of cell migration in response to a host of interventions, including stimulation of GPCRs. Migration of J82 carcinoma cells is stimulated by LPA and thrombin but not by bradykinin, bombesin, and histamine, other G_q-coupled agonists (122). Responses to both LPA and thrombin were inhibited by C3 exoenzyme. We reported that migration of vascular smooth muscle cells was likewise induced by thrombin, but not phenylephrine, in a C3-sensitive manner (46). Using Y27632, we suggested the involvement of Rho kinase in GPCR-stimulated vascular smooth muscle cell migration. Consistent with these observations, Kaibuchi's laboratory showed that microinjection of dominant negative Rho kinase inhibited migration of NRK49F cells in a wound

healing assay (123). Y27632 was also shown to inhibit chemotactic peptide-(fNLPNTL) stimulated migration of human neutrophils (124).

Phosphorylation of cytoskeletal-associated proteins such as FAK, paxillin, MLC, and α-adducin have been associated with cell migration, and phosphorylated forms of both α-adducin and MLC have been observed at the leading edge of migrating cells (123, 125). Consistent with involvement of myosin phosphorylation in cell migration, microinjection of an antibody to MLC phosphorylated at Ser^{19} (the site of phosphorylation by Rho kinase and MLCK) inhibited fNLPNTL-stimulated migration of human neutrophils (124). It was further demonstrated that Rho kinase can phosphorylate α-adducin and that a mutant form of α-adducin, which cannot be phosphorylated, was able to inhibit migration of NRK49F cells (123). Therefore, Rho kinase-mediated phosphorylation of α-adducin and MLC appears to be important in regulating cell migration.

Studies investigating biochemical pathways involved in cancer cell invasion, a critical event in malignant metastasis, have also indicated a role for GPCRs and Rho. Early studies of cell invasion revealed that rat MM1 hepatoma cells could penetrate a cell monolayer in the presence of serum in vitro. It was later found that the response to serum could be fully reproduced by LPA (126). Studies using C3 exoenzyme have implicated Rho as a necessary signal transducer of LPA-stimulated cell invasion (127). In addition, expression of activated RhoA enhanced LPA-mediated MM1 cell invasion, consistent with a potential positive feedback loop involved in Rho activation (128).

MAP Kinase Activation and Gene Transcription

On activation by extracellular stimuli, the MAP kinase family of serine/threonine kinases phosphorylate transcription factors, increasing their transcriptional activity and thereby regulating gene expression. Receptor tyrosine kinases and GPCRs that activate the small G proteins Ras or Rac initiate kinase cascades leading to MAP kinase activation. The ability of Rho family proteins to activate MAP kinase cascades has also been examined. Rho alone is not sufficient to activate extracellular signal-regulated kinase (ERK) (129, 130, 130a), although it can cooperate with and enhance other stimulatory signals that lead to ERK activation (130, 131). In addition, whereas activated forms of Rac and Cdc42 are potent stimulators of c-Jun N-terminal kinase (JNK) and p38, RhoA is ineffective in the same assays (129, 130, 130a, 132, 133). Furthermore, although dominant interfering mutants of Rac and Cdc42 attenuated JNK activation, dominant negative RhoA had no effect (129, 132). A paper by Teramoto et al (134), however, reported that constitutively active RhoA, -B, or -C stimulated JNK in 293T cells (134). Paradoxically, it has also been noted that C3 stimulates JNK and p38 in Rat-1 cells, possibly due to stress activation of these kinases (37a). Although some cell type–specific effects of Rho on MAP kinases may exist, these kinases do not appear to be the primary downstream targets of Rho activation.

A role for Rho in transcriptional regulation was first demonstrated by Hill et al (39). Their work established that activated RhoA stimulates gene expression through the c-fos serum response element (SRE). SRE sites are regulated by serum response factor (SRF) acting in conjunction with ternary complex factor. Hill et al showed that RhoA stimulated the transcriptional activity of several mutants of the c-fos SRE including one (SRE.L) that has a high affinity-binding site for SRF but does not bind ternary complex factor. A variety of GPCRs stimulate transcription from the SRE.L, and these responses are largely inhibited by C3 exoenzyme or dominant negative RhoA (6, 8, 39).

Constitutively active mutants of the Rho effectors, Rho kinase and PRK2 have been shown to weakly activate SRE-mediated gene expression (66, 70, 135). Studies utilizing mutant constructs of activated RhoA identified three residues in the effector loop that are vital for activating SRF-mediated gene expression (136). However, the loss of SRF activation could not be correlated with the loss of binding of Rho to any known effector (66). It is interesting to note that these effector loop mutants were able to dissociate the ability of RhoA to induce SRF and to induce stress fibers, indicating that different effectors (or different combinations of effectors) mediate these two responses (66). Y27632 failed to block c-fos SRE activation, further indicating that SRF activation is not dependent on Rho kinase activity (137).

Nuclear factor (NF) κB plays a key role in immune function, inflammation, and lymphoid differentiation. GPCR agonists such as bradykinin and LPA have been demonstrated to regulate NFκB transcriptional activation (138–140). Activation of an NFκB reporter gene by bradykinin was inhibited by dominant negative RhoA or C3 exoenzyme and mimicked by constitutively active RhoA (9). A possible mechanism by which RhoA mediates NFκB activation is by enhancing the phosphorylation of IκBα, which leads to IκBα degradation and the subsequent nuclear translocation of NFκB. Montaner et al (141) found that a nonphosphorylatable mutant of IκBα prevented NFκB activation by Rho. Of particular note, the ability of Rho and Rho-specific GEFs to stimulate the SRE.L was also inhibited, which suggests that NFκB- and SRE-mediated gene expression might be interdependent.

Some recent reports have shown an involvement of Rho in AP-1–mediated transcription. Chang et al (142) reported that activated RhoA potentiated phorbol ester-induced AP-1–mediated gene expression in T cells, which suggests that RhoA- and PKC-mediated pathways interact. This was suggested to occur through the binding of RhoA to PKCα, because expression of the N terminus of PKCα prevented this effect of Rho (142). Studies from our lab also suggest the involvement of Rho in thrombin-induced AP-1 activation (SA Sagi, S Schubbert & JH Brown, unpublished observations). Expression of N19RhoA inhibited thrombin-induced AP-1–luciferase expression in 1321N1 astrocytoma cells, whereas activated RhoA or a RhoGEF (Lbc) mimicked the effect of thrombin. Although the full extent of Rho involvement in AP-1–mediated gene transcription is unknown, further investigation is warranted.

Cell Growth and Survival Responses

GPCR-induced signals can stimulate proliferative cell growth. Indeed, activation of a number of these serpentine receptors has been shown to display mitogenic effects and to have transforming potential (reviewed in 143, 144). Aberrant cell growth has also been observed with mutationally activated mutants of various G protein α subunits, including those of $G\alpha_i$, $G\alpha_q$, $G\alpha_{12}$, and $G\alpha_{13}$ (reviewed in 143). A role for Rho in regulating cell proliferation was first suggested by studies in which it was demonstrated that C3-mediated inhibition of Rho caused fibroblasts to arrest in the G_1 phase of the cell cycle (145). Inhibition of protein geranylgeranylation also results in G_0/G_1 cell cycle arrest. This arrest has been attributed to blockade of Rho function, because newly synthesized RhoA is geranylgeranylated and translocates to the membrane fraction during G_1-S progression in growth-stimulated cells (146, 147). Consistent with the observed involvement of Rho in cell cycle progression, the incorporation of the thymidine analog, bromodeoxyuridine, into nascent DNA (an indicator of G_1-S progression) was stimulated by microinjection of GTPase-deficient RhoA into quiescent fibroblasts (130a). The finding that Rho is required for serum- and Ras-induced DNA synthesis may be explained by the ability of activated Rho to stimulate the degradation of cyclin-dependent kinase inhibitors, thereby permitting G_1-S progression and DNA synthesis (147a). DNA synthesis induced by thrombin and endothelin-1 has been reported to be C3 sensitive, which suggests a role for Rho in GPCR-stimulated cell proliferation (46, 78, 148a).

Although RhoA alone displays weak transforming ability, it can strongly cooperate with the Ras-Raf pathway in focus formation (7, 148). When constitutively activated mutants of RhoA and Ras were coexpressed, a synergistic enhancement in transforming activity was observed, and a dominant negative RhoA mutant reduced oncogenic Ras-induced transformation (7, 148). Similarly, we observed that although expression of activated RhoA alone was insufficient to induce DNA synthesis, it acted synergistically with activated Ras (46). The involvement of Rho kinase in cellular transformation has been implicated through studies utilizing Y27632 and Rho effector domain mutants (66, 136, 137).

The muscle cells of the heart are terminally differentiated at birth. In response to growth-promoting signals, these cells undergo hypertrophy, a phenomenon whereby cell size is increased without increased cell number. Cardiomyocyte hypertrophy is characterized by the induction of a specific subset of genes, including that of c-fos, atrial natriuretic factor, MLC 2, and skeletal α-actin and by the organization of sarcomeric proteins into contractile units. In cultured neonatal rat cardiomyocytes, these hypertrophic responses are elicited through stimulation of receptors coupled to G_q, including the α_1-adrenergic, endothelin, prostaglandin $F_{2\alpha}$, and angiotensin II receptors. Work from our laboratory and that of others has shown that GPCR agonist-induced hypertrophic gene expression and actin myofibrillar organization are RhoA-dependent events (47, 62, 129, 149–151). Additional data suggest that RhoA is a downstream mediator of $G\alpha_q$ signaling in a

pathway that acts synergistically with that activated by Ras (129, 150). Rho kinase was implicated as a mediator of hypertrophy, because inhibitory mutants of Rho kinase and Y27632 were able to attenuate the hypertrophic responses triggered by constitutively active RhoA and endothelin (62, 151).

To study the role of RhoA in regulating cardiac function in vivo, transgenic mice with cardiac-specific expression of wild-type or activated forms of RhoA were generated. It is surprising to note that these mice did not manifest cardiac hypertrophy but instead had greatly reduced survival rates due to the development of severe bradycardia, conduction system disturbances, and left ventricular contractile dysfunction (152). RhoA has been demonstrated to associate with and suppress the activity of a delayed rectifier K^+ channel Kv1.2 (153). It was suggested, therefore, that the phenotype seen in the transgenic mice could result from effects of RhoA on K^+ channel function, either directly or through changes in the actin cytoskeleton.

GPCR agonists not only elicit growth responses, they can also activate cell death. Constitutively active mutants of G protein α subunits, including those of the $G_{q/11}$ and $G_{12/13}$ families, have been shown to trigger programmed cell death (apoptosis) when heterologously expressed in COS-7 cells or cardiomyocytes (154–156). Although constitutively activated $G\alpha_q$ was shown to induce apoptosis through a PKC-dependent mechanism, $G\alpha_{13}$ did so via a RhoA-dependent pathway (154). In cardiomyocytes, a low level of $G\alpha_q$ promoted cell growth whereas excessive activation induced apoptosis (156). Similarly, in neuronal and astroglial cells, the GPCR agonist thrombin is neuroprotective at moderate concentrations (157), but at higher concentrations, it induces apoptosis (45). The ability of thrombin to protect from hypoglycemia and induce apoptosis was attenuated by C3 exoenzyme treatment, suggesting that Rho can participate in both cell protection and cell death (45, 158).

Several mechanisms through which GPCRs regulate cell survival and apoptosis have been proposed. For example, it has been shown that m_1 and m_2 muscarinic receptor stimulation can lead to phosphorylation and activation of a serine/threonine protein kinase Akt/PKB (159). Akt/PKB is regulated through PI(3)K and synthesis of PI(3,4)P_2 and PI(3,4,5)P_3 (99). Thus, it is possible that GPCRs utilize Rho to regulate this cell survival pathway. Rho activation has also been reported to regulate dynamic membrane blebbing (a process that may be mediated by MLC phosphorylation) during the final stages of apoptotic cell death (160). The Rho effector PKN is another possible mediator of apoptosis, because it is proteolytically cleaved by caspases to generate a constitutively activated kinase fragment (161). However, there is as yet no direct evidence implicating specific signaling molecules or pathways downstream of Rho in the apoptotic signaling cascade.

Other Responses

In addition to the numerous Rho-dependent cellular processes described above, Rho function has also been implicated in the regulation of endocytosis, exocytosis, glucose transport, and ion channels. Internalization of m_1 and m_2 mAChRs,

via clathrin-coated pit-dependent and -independent mechanisms, respectively, was inhibited by overexpression of wild-type RhoA, although Rho did not appear to be an endogenous mediator of mAChR sequestration (162). It had previously been noted that activated RhoA inhibits clathrin-coated vesicular endocytosis (163) and that Rho is a mediator of the effects of Gβγ on clathrin-dependent endocytosis (164). In chromaffin cells, mastoparan-mediated activation of G_o inhibits Ca^{2+}-induced disassembly of the actin network and accompanying exocytotic catecholamine secretion (165). These effects of mastoparan are inhibited by C3 exoenzyme, which suggests that the regulatory effect of G_o on exocytosis requires Rho, possibly via its effects on the actin cytoskeleton.

Glucose transport is an early cellular response to growth factors and is essential for cell proliferation. Several reports suggest a role for Rho in regulating glucose transport (166, 167). For instance, LPA-stimulated deoxyglucose uptake was shown to be inhibited by C3 exoenzyme (166). Furthermore, GTPγS-induced GLUT4 translocation and glucose transport were inhibited by C3 exoenzyme and dominant negative forms of RhoA and PKN (167). Thus, RhoA-dependent pathways appear to be required for the regulation of glucose transport.

A role for Rho in regulating ion channel function has been demonstrated by Cachero et al (153). These investigators initially showed that stimulation of the m_1 mAChR resulted in a tyrosine kinase-dependent suppression of the basal K^+ current generated by the delayed rectifier, Kv1.2 ($I_{Kv1.2}$). A yeast two-hybrid screen identified RhoA as a Kv1.2-interacting protein. Overexpression of RhoA was shown to mimic the effects of carbachol on $I_{Kv1.2}$, and this appeared to be dependent on the physical interaction between RhoA and Kv1.2. In addition, C3 exoenzyme blocked the carbachol-mediated tyrosine kinase-dependent suppression of Kv1.2 (153), demonstrating a role for RhoA in the modulation of GPCR-mediated Kv1.2 activity. These provocative data suggest the possibility that additional ion channels will be found to be regulated through Rho-dependent mechanisms.

MECHANISMS OF RHO ACTIVATION

Pertussis Toxin Sensitivity

Most of the GPCR agonists that regulate actin cytoskeletal responses, smooth muscle contraction, gene transcription, and cell growth through Rho-dependent pathways can couple to more than a single class of heterotrimeric G proteins. For example LPA and thrombin elicit cellular responses through both pertussis toxin–sensitive and –insensitive G proteins (74, 168, 169, 176). GPCR agonist-induced stress fiber formation, focal adhesion complex assembly, and cell rounding are generally pertussis toxin–insensitive (76, 78, 170–171a). Comparison of the effects of microinjected activated Gα subunits demonstrated negligible cytoskeletal effects of $G\alpha_i$ relative to those of the pertussis toxin–insensitive G proteins $G_{q/11}$ and $G_{12/13}$ (172, 173). Furthermore, expression of activated $G\alpha_i$ in COS-7

cells produced no increase in the level of activated Rho (54). These observations indicate that $G_{i/o}$ proteins are not sufficient or necessary for GPCR-mediated activation of Rho or Rho-dependent cytoskeletal responses.

There are exceptions to this pattern, however. For example, stimulation of heterologously expressed α_2-adrenergic receptors in preadipocytes led to Rho-mediated changes in cell morphology and increases in FAK phosphorylation. These responses were shown to be sensitive to pertussis toxin but not to $G\beta\gamma$ sequestration by the βARK1 C-terminal domain and therefore appeared to be mediated through the $G\alpha_{i/o}$ subunit (52). Increases in membrane association of and [^{32}P]GTP binding to Rho were also observed in response to α_2-adrenergic receptor stimulation in this system. In addition, LPA-mediated increases in membrane-associated Rho in Swiss 3T3 fibroblasts and migration of J82 carcinoma cells were pertussis toxin–sensitive (44, 122). Paradoxically, βγ subunits isolated from $G_{i/o}$ were shown to bind to Rho and inhibit Rho-GTPγS binding, which suggests the opposite, i.e. a possible inhibitory effect of $G_{i/o}$ proteins on Rho (174).

Regulation by $G_{q/11}$

Most of the GPCR agonists shown to activate Rho are coupled to G_q-mediated pathways. In spite of this, considerable evidence suggests that G_q-mediated signaling pathways are not sufficient as regulators of Rho-mediated cytoskeletal and other responses. First, it is clear that not all receptors that couple to G_q and activate PLC are able to elicit Rho-dependent cytoskeletal responses, cell migration, or DNA synthesis (46, 78, 79, 122, 175, 176). For example, cell rounding is elicited by thrombin but not by carbachol in 1321N1 and N1E115 cells (78, 175), and by LPA but not bradykinin in PC12 cells (79). Second, G_q/PLC-generated second messenger pathways (Ca^{2+}, PKC) are not sufficient (76, 79) or required for cytoskeletal responses to GPCR agonists such as thrombin and LPA (78, 171a, 175). In addition, recent studies used platelets and fibroblast cell lines derived from wild-type and $G\alpha_q$/$G\alpha_{11}$-deficient mice to unequivocally demonstrate that activation of several receptors that can couple to $G_{q/11}$ (including those for thrombin, LPA, thrombaxane A_2, and endothelin) induce shape changes even in the absence of $G\alpha_q$ and $G\alpha_{11}$ (92, 176). Consistent with this, $G\alpha_q$ antibodies only weakly inhibit the Rho-dependent effects of LPA and thrombin on the cytoskeleton (19, 53).

On the other hand, a signaling pathway dependent on Ca^{2+} and PKC leads to neurite retraction in response to expression of constitutively activated $G\alpha_q$ in PC12 cells (173). Furthermore, heterologously expressed m_1 muscarinic and metabotropic glutamate receptors induce Rho-dependent stress fiber formation in mouse fibroblasts, and this response is abolished in fibroblasts from $G\alpha_{q/11}$-deficient mice (176). This finding implies that coupling of $G_{q/11}$ to at least some GPCRs is required for the cytoskeletal response (176). Rho-dependent regulation of the SRE.L by GPCR agonists, as described above, can also be induced by expression of activated $G\alpha_q$ and through heterologously expressed m_1 mAChRs

or α_1-adrenergic receptors in wild-type but not G_q-deficient cells (8, 18). Thus, heterologously expressed G_q-coupled receptors or GTPase-deficient $G\alpha_q$ can activate signaling pathways that contribute to Rho activation or its ability to elicit downstream responses, although the contribution of these pathways to endogenous signaling is not clear.

Regulation by $G_{12/13}$

Recent evidence indicates that activation of Rho and its downstream effectors is primarily mediated through G proteins of the $G_{12/13}$ family. $G_{12/13}$ proteins, isolated as oncogenes and cloned by homology to other G proteins, have been unique in their failure to regulate known Gα effectors such as adenylyl cyclase or phospholipases (reviewed in 177). The ability of activated $G\alpha_{12/13}$ subunits to induce stress fiber formation was first demonstrated by Buhl et al in studies using 3T3 fibroblasts (172). Constitutively active forms of $G\alpha_{12}$ and $G\alpha_{13}$ have subsequently been shown to induce stress fiber and focal adhesion formation, as well as tyrosine phosphorylation of FAK and paxillin, in a Rho-dependent manner (53, 180). Both $G\alpha_{12}$ and $G\alpha_{13}$ also induce neurite retraction and cell rounding when expressed in PC12, N1E-115, or 1321N1 cells (19, 54, 173). In addition, activated $G\alpha_{12}$ and $G\alpha_{13}$ cause transcriptional activation of the SRE.L reporter in a Rho-dependent manner (6, 8, 17, 18, 181).

The ability of these constitutively active G protein α subunits to induce the aforementioned responses suggests, but does not prove, their involvement in agonist-mediated responses. This question has been addressed by microinjection of G protein C-terminal antibodies, which block receptor–G protein coupling. Experiments carried out in several laboratories demonstrate that thrombin and LPA effects on the cytoskeleton can be blocked by antibodies to $G\alpha_{12}$ and $G\alpha_{13}$ (19, 53). Both of these receptors have been shown by GTP-labeling studies to couple to $G\alpha_{12}$ and $G\alpha_{13}$ (178, 179). Specificity in receptor coupling to $G\alpha_{12}$ vs $G\alpha_{13}$ was suggested by the microinjection experiments (19, 53) and confirmed by the use of inhibitory mutants of $G\alpha_{12}$ and $G\alpha_{13}$ and by the use of $G\alpha_{13}$-deficient fibroblasts (176). The results of these studies suggest that the LPA receptor functions primarily through G_{13} and the thrombin receptor primarily acts through G_{12}.

Involvement of Tyrosine Kinases

It is interesting to note that the pathways by which G_{12} and G_{13} signal to Rho also appear distinct. Early studies had suggested that a tyrphostin A25–sensitive tyrosine kinase functioned upstream of Rho in agonist-induced cytoskeletal pathways (182). In a recent report, tyrphostin A25 was shown to inhibit $G\alpha_{13}$-induced morphological changes in PC12 cells (173), as well as LPA- and $G\alpha_{13}$-induced stress fibers and focal adhesion assembly in 3T3 cells (53), but failed to block the effects of $G\alpha_{12}$ in the same systems. In addition, tyrphostin AG1478, an epidermal growth factor (EGF) receptor-specific tyrosine kinase inhibitor, blocked cytoskeletal responses induced by $G\alpha_{13}$ and LPA but not those induced by throm-

bin or $G\alpha_{12}$ (53, 176). Thus, tyrosine kinases and the EGF receptor have been implicated in the $G\alpha_{13}$ but not $G\alpha_{12}$ pathways.

Two groups have now independently shown that expression of activated forms of either $G\alpha_{12}$ or $G\alpha_{13}$ in COS-7 cells increases Rho activation, as measured by increases in [^{32}P]GTP-binding to Rho (53) and increases in Rho binding to the GST-RBD of Rho kinase (54). Results of these studies also suggest that the EGF receptor tyrosine kinase is upstream of Rho, i.e. involved in the pathway by which $G\alpha_{13}$ leads to Rho-GTP loading (53). Tyrosine kinase involvement in LPA-mediated Rho activation was further substantiated by a study demonstrating inhibition of LPA-stimulated Rho-RBD binding by pretreatment with either genistein or tyrphostin 47 (54).

The EGF receptor is not the only tyrosine kinase that affects Rho activation. Nonreceptor tyrosine kinases of the Tec/Bmx family may be involved in $G\alpha_{12/13}$-induced Rho and SRE.L activation (181). Transfection of Tec into COS-7 cells was shown to increase membrane-associated Rho indicative of Rho activation. This family of tyrosine kinases was also suggested to function in response to $G\alpha_{12/13}$, because synergistic activation of the SRE.L was observed when activated $G\alpha_{13}$ and Tec or Bmx were coexpressed, and because constitutively active $G\alpha_{13}$ was shown to increase the tyrosine phosphorylation and activation of Tec. In addition, thrombin-induced SRE.L activation in $G_{q/11}$-deficient cells was shown to be inhibited by a kinase-dead mutant of Tec (181). Other recent evidence suggests that calpeptin may prevent Rho activation through inhibition not only of calpeptin, but also of membrane-associated tyrosine phosphatase activity (183, 184). Thus, it appears likely that tyrosine phosphorylation of an as-yet-unidentified signaling molecule(s) plays a key role in the control of Rho activation.

Involvement of RhoGEFs

The most exciting development concerning mechanisms by which GPCRs and $G\alpha_{12/13}$ activate Rho is the discovery that $G\alpha_{12/13}$ family proteins can interact directly with and activate RhoGEFs. Two papers describe this novel and important regulatory pathway (25, 185). The authors observed that the p115RhoGEF contained an RGS-like domain at its N terminus and demonstrated that this domain was required for binding to $G\alpha_{12}$ and $G\alpha_{13}$. Significantly, they showed that p115RhoGEF, like other RGS proteins and Gα protein effectors, served as a GAP for both $G\alpha_{12}$ and $G\alpha_{13}$. The finding of most fundamental importance, however, was that $G\alpha_{13}$ stimulated p115RhoGEF activity, providing a direct mechanism by which the $G\alpha_{13}$ protein could induce Rho activation. It is also of interest that although p115RhoGEF bound to and acted as a GAP for $G\alpha_{12}$, its activity as a GEF was not activated by $G\alpha_{12}$. This is consistent with work cited above that suggests that $G\alpha_{12}$ and $G\alpha_{13}$ activate Rho through different mechanisms. Mao et al (18) used the SRE.L reporter gene in transient transfection assays to demonstrate that $G\alpha_{13}$ synergizes with p115RhoGEF and thus presumably acts through p115RhoGEF to activate SRE.L. They further demonstrated that a mutant p115RhoGEF lacking the DH domain served as a dominant negative inhibitor of

LPA and thrombin effects on the SRE.L. These data are significant in that they implicate this or similar RhoGEFs in Rho-mediated agonist-induced gene transcription. The PDZ-RhoGEF, analyzed by Fukuhara et al (17), was also shown to bind to both $G\alpha_{12}$ and $G\alpha_{13}$. A mutant PDZ-RhoGEF with the DH and PH domains deleted blocked both $G\alpha_{12/13}$-and LPA-mediated activation of the SRE.L. Recent work from our laboratory demonstrated that $G\alpha_{12/13}$ mediate the effects of thrombin on the cytoskeleton in 1321N1 cells and that inactive forms of either p115RhoGEF or Lbc inhibit cell rounding induced by either $G\alpha_{12}$ or thrombin (19).

Although much of the work above indicates that $G\alpha_{12/13}$ can interact directly with RhoGEFs, it is likely that second messenger pathways and kinase cascades also regulate Rho activity. For instance, activation of phospholipase A_2 by Rac was shown to increase arachidonic acid and, subsequently, activation of the SRE.L reporter gene (186). This was blocked by dominant negative RhoA or a C3 expression plasmid, indicating a potential role for PLA_2 signaling in Rho activation. Activation of PI(3)K in response to serum also leads to Rho-mediated c-fos SRE activation (187). RhoGAPs can be phosphorylated by protein kinases such as Src and MAP kinase (11, 13, 14), and GDIs contain sequences for phosphorylation by casein kinase II, PKC, and cGMP-dependent protein kinase (29). Phosphorylation of RhoGEFs has not to our knowledge been described, but the RacGEF Tiam has been shown to be phosphorylated by PKC in response to LPA (187a). There is also evidence that cAMP and cAMP-dependent protein kinase can affect Rho activation. Agonist-stimulated [^{35}S]GTPγS binding to Rho was shown to be inhibited by 8-bromo-cAMP (51). In addition, cAMP-dependent protein kinase phosphorylates Rho; this has been demonstrated to be associated with increases in cytosolic Rho (188) and to inhibit the ability of Rho to alter cell morphology and bind Rho kinase (189). Thus phosphorylation of Rho or its regulators (GAPs, GDIs, or GEFs) is likely to provide additional mechanisms by which GPCRs can modulate Rho signaling pathways.

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