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MINIREVIEW

Rho as a Mediator of G Protein-Coupled Receptor Signaling

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Heterotrimeric GTP-binding proteins (G proteins) contain α subunits that switch between an inactive GDP-bound state and an active GTP-bound state in response to agonist binding to heptahelical receptors. The low-molecular-weight or small G proteins are also GTPases that serve as molecular switches. However, their activation is not directly regulated through interaction with agonist-bound G protein-coupled receptors (GPCRs). Instead, GTP exchange on the small G proteins is controlled through guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP.

Low-molecular-weight G proteins in both the Ras and Rho (Rho, Rac, and Cdc42) subfamilies have been demonstrated to play critical roles in growth regulation and in control of the actin cytoskeleton. There is now considerable evidence that GPCR activation can regulate cell growth and induce actin cytoskeletal rearrangement, and that these responses are mediated, at least in part, through the engagement of the low-molecular-weight G proteins. The discovery of the central role of a specific GEF (son of sevenless) in the pathway for Ras activation defined a new paradigm in signaling from cell-surface receptors to kinase cascades. Significantly, it established the concept that GEFs can be regulated through extracellular signals.

This review will focus specifically on the involvement of the low-molecular-weight G protein RhoA in mediating responses to GPCRs. Factors known to regulate Rho activation and interventions used to modulate Rho function will be reviewed briefly, as will the evidence that a range of GPCR-induced responses require Rho. We will then consider evidence that Rho can be activated by agonist stimulation of GPCRs and discuss recent evidence that the control of GEF activity is one possible molecular mechanism by which this occurs.

Modulators of Rho Function

Under unstimulated conditions, the major cellular fraction of Rho is found in the cytosol bound to guanine nucleotide dissociation inhibitors (GDIs) specific for the Rho family of small GTPases (Sasaki and Takai, 1998). These inhibitory proteins bind to the carboxyl terminus of Rho, extracting it from membranes and inhibiting GTPase cycling. GTPase-activating proteins (GAPs) regulate the inactivation of G proteins by accelerating their intrinsic GTPase activity. A number of GAPs that can interact with and have specificity for Rho have been identified. These include Graf, which can be phosphorylated by mitogen-activated protein kinase on serine510 and which colocalizes with the actin cytoskeleton (Taylor et al., 1998), and p122-RhoGAP, which has been shown to bind to and activate phospholipase C $(PLC)_{\delta}$ (Homma and Emori, 1995). GEFs mediate the activation of small GTPases by catalyzing the exchange of GDP for GTP. A family of Rho GEFs including lbc, lsc, and lfc was first identified as oncogenes (Toksoz and Williams, 1994; Glaven et al., 1996). A Dbl-homology (DH) domain responsible for exchange activity and a Pleckstrin-homology domain thought to be involved in subcellular localization are common to GEFs. The p115-RhoGEF and another newly discovered GEF homolog (PDZ-RhoGEF) not only have Pleckstrin-homology and DH domains but also possess regions with homology to regulators of G protein-signaling proteins, potential sites for interaction with heterotrimeric G proteins (Hart et al., 1998; Kozasa et al., 1998; Mao et al., 1998; Fukuhara et al., 1999).

Tools for Examining Rho Function

The C3 exoenzyme, one of a number of toxins isolated from *Clostridium botulinum*, has been a valuable probe for analyzing Rho involvement in various cellular functions. The C3

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ABBREVIATIONS: G protein, GTP-binding protein; GPCR, G protein-coupled receptor; GEF, guanine nucleotide exchange factor; GDI, guanine nucleotide dissociation inhibitor; GAP, GTPase activating protein; LPA, lysophosphatidic acid; MLC, myosin light chain; p160ROCK/Rho kinase, Rho-dependent kinase; SRE, serum response element; SRF, serum response factor; mAChR, muscarinic cholinergic receptor; αAdrR, α-adrenergic receptor; PLC, phospholipase C; PKC, protein kinase C; ($i_{KV1.2}$), delayed rectifying potassium channel; PKA, protein kinase A; DH, Dbl-homology domain; cAMP, cyclic AMP.

exoenzyme has been shown to specifically ADP-ribosylate Rho at residue Asn[41] in its effector domain, rendering it inactive (Sekine et al., 1989; Yamamoto et al., 1993). Cellular delivery is best achieved through the microinjection of the C3 protein (Gohla et al., 1998; Katoh et al., 1998) or expression of the C3 exoenzyme (Hill et al., 1995b; Sah et al., 1996; Needham and Rozengurt 1998), but the C3 exoenzyme also has been effectively applied extracellularly (Nishiki et al., 1990; Yamamoto et al., 1993; Majumdar et al., 1998). Cytotoxic necrotizing factor-1, isolated from *Escherichia coli*, was recently shown to specifically deamidate Gly63 in Rho to Glu, resulting in Rho activation (Fiorentini et al., 1997; Flatau et al., 1997; Schmidt et al., 1997), accounting for the ability of this factor to mimic at least some of the effects of Rho when added to cells. Constitutively activated or dominant interfering mutants of RhoA also have been generated. The substitution of asparagine for serine at position 19 results in a protein (N19RhoA) that has a decreased affinity for GTP and an increased affinity for RhoGEFs and, hence, acts as a competitive inhibitor of endogenous Rho activation. The substitution of valine for glycine at position 14 (V14RhoA) or of leucine for glycine at position 63 (L63RhoA) abolishes GTPase activity and results in a constitutively active form of Rho. Several RhoGEFs isolated as oncogenes also appear to be constitutively active; these have been commonly used to induce Rho-dependent responses (Zheng et al., 1995; Hart et al., 1996; Barrett et al., 1997; Hart et al., 1998; Kozasa et al., 1998). Most recently, several mutant RhoGEFs lacking exchange activity have been demonstrated to act as dominant negative inhibitors (Mao et al., 1998; Fukuhara et al., 1999; M.M., C. Buckmaster, D. Toksoz, T.M.S., and J.H.B., in preparation) of agonist- or G protein-mediated responses.

Rho Involvement in GPCR-Induced Cytoskeletal Rearrangement

When activated RhoA is microinjected into fibroblasts, actin fibers organize to form filamentous structures termed stress fibers. The assembly of stress fibers is accompanied by the formation of focal adhesion plaques, regions serving to transduce signals from the extracellular matrix to tyrosine kinases and other signaling proteins localized within the focal adhesion. Hall's laboratory (Ridley and Hall, 1992) demonstrated that the addition of serum to starved Swiss 3T3 cells led to the rapid induction of stress fiber formation and suggested that lysophosphatidic acid (LPA) was the mediator of this serum response. The ability of LPA to induce stress fibers appeared to be Rho dependent, as it was inhibited by the C3 exoenzyme (Ridley and Hall, 1992). p125FAK and paxillin, prominent proteins localized in the focal adhesions, are tyrosine phosphorylated in response to serum stimulation, as well as in response to LPA, bombesin, and endothelin (Kumagai et al., 1993; Rankin et al., 1994; Ridley and Hall, 1994; Seckl et al., 1995). The addition of GTPγS to permeabilized cells (Seckl et al., 1995) also stimulates tyrosine phosphorylation of these proteins, and C3 pretreatment prevents the agonist- or GTPγS-induced responses (Rankin et al., 1994; Seckl et al., 1995). These pioneering studies establish that the activation of certain GPCRs induces Rho-dependent stress fiber formation, focal adhesion formation, and tyrosine kinase activation.

In neuronal, PC-12, and astroglial cells, GPCR agonists, including LPA, sphingosine-1-phosphate, prostaglandins, and thrombin, evoke a very different type of actin cytoskeletal response, which is characterized by rounding of the cell body and retraction of cell processes (Jalink et al., 1994;

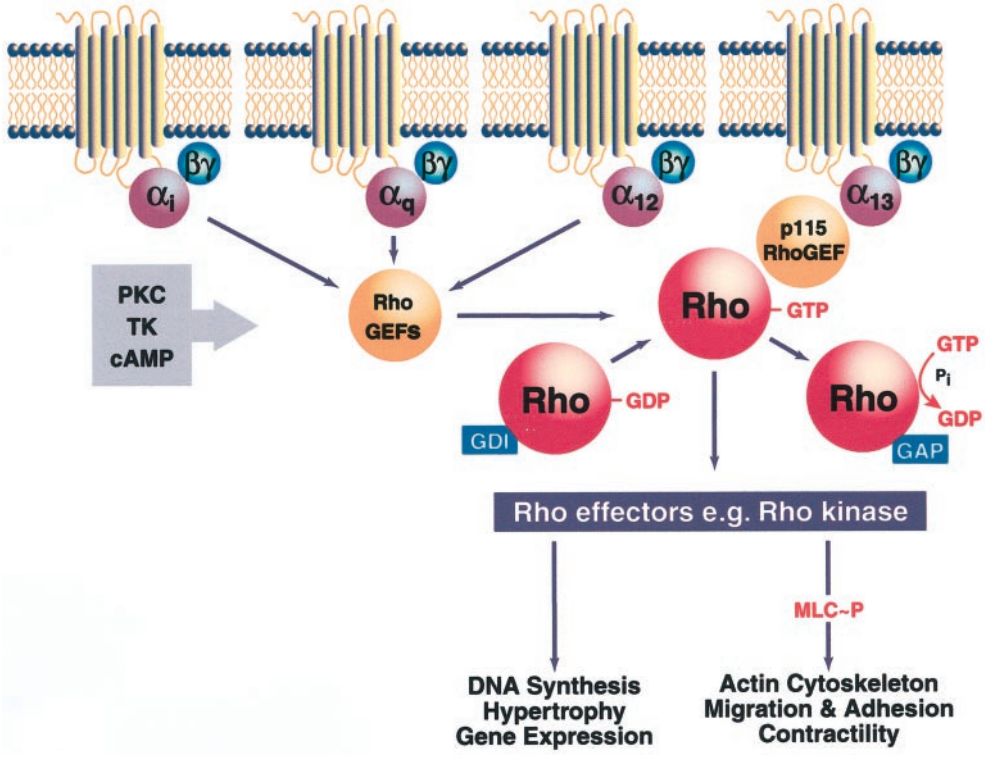

Fig. 1. Proposed signaling pathways for GPCR activation of Rho. The α or $\beta\gamma$ subunits from G_i and the α subunits from G_q and G_{12} are postulated to activate Rho by regulating RhoGEFs. Neither the mechanism for RhoGEF activation nor the identity of these RhoGEFs has been ascertained. Direct interactions between G protein subunits and RhoGEFs are possible, as are indirect effects mediated via PKC, tyrosine kinases (TK) or cAMP. For $G_{\alpha 13}$, a direct interaction with a specific RhoGEF, p115-RhoGEF, is known to enhance exchange activity and Rho-GTP binding. Other Rho regulatory proteins that are possible sites of modulation by GPCR signaling pathways include RhoGAPs, which accelerate Rho inactivation, and Rho GDP GDIs, which maintain unstimulated Rho in a cytosolic GDP-bound state. Rho binds to and activates numerous effectors, the best-characterized of which are members of the Rho kinase family. Rho kinase increases the extent of MLC phosphorylation, contributing to actin cytoskeletal rearrangement, cell migration, and adhesion and contractility. Responses for which the Rho effector is less clearly identified include DNA synthesis, hypertrophic growth, and gene expression.

Katoh et al., 1996; Postma et al., 1996; Tigyi et al., 1996b; Majumdar et al., 1998). Moolenaar's laboratory (Jalink et al., 1994) observed this response in N1E-115 and NG108-15 cells stimulated with LPA and thrombin peptide and demonstrated that it was C3 sensitive. Interestingly, some, but not all, PLC-coupled receptor agonists induce cell rounding. LPA, but not bradykinin, is effective in PC12 cells (Tigyi et al., 1996b), and thrombin, but not carbachol, is effective in 1321N1 astroglial cells (Majumdar et al., 1998). Stress fiber formation also has been dissociated from the activation of G_q, PLC, Ca^{++} mobilization, and protein kinase C (PKC; Ridley and Hall, 1994; Buhl et al., 1995; Seckl et al., 1995). Evidence that G proteins of the $G_{12/13}$ family (rather than or in addition to the G_q family) are responsible for induction of these cytoskeletal responses is discussed later in this review.

In addition to stress fiber formation, cell rounding, and process retraction, GPCRs can regulate cell adhesion and migration through Rho-dependent processes. For example, Rho-dependent changes in cell motility are induced by formylmethionylleucylphenylalanine in leukocytes (Laudanna et al., 1996), by thrombin in vascular smooth muscle cells (Seasholtz et al., 1999), and by LPA in tumor cells (Yoshioka et al., 1998).

The mechanisms by which Rho activation induces changes in the actin cytoskeleton are under intensive investigation and are beyond the scope of this review. Briefly, there is considerable evidence that myosin light chain (MLC) phosphorylation is regulated through a Rho-dependent kinase (p160ROCK/Rho kinase) that can phosphorylate and functionally inhibit the myosin-binding subunit of myosin phosphatase (Kimura et al., 1996) and, perhaps, directly phosphorylate MLC (Amano et al., 1996). Actin-myosin-mediated contractile events are postulated to contribute to the LPA- and thrombin-mediated formation of stress fibers (Chrzanowska-Wodnicka and Burridge, 1996), cell rounding (Jalink et al., 1994; Buhl et al., 1995; Essler et al., 1998; Hirose et al., 1998; Majumdar et al., 1998), and cell migration (Yoshioka et al., 1998). Activation of the Na^+/H^+ antiporter also has been implicated as a mediator of Rho- and Rho kinase-dependent stress fiber formation in fibroblasts (Vexler et al., 1996; Tominaga et al., 1998). The ERM family of actin-binding proteins, including ezrin, radixin and moesin, also have been shown to be required for Rho- and Rho kinase-dependent cytoskeletal rearrangements (Fukata et al., 1998).

Rho Involvement in Ca^{++} Sensitization and Vascular Smooth Muscle Contraction

The classic pathway responsible for vascular smooth muscle contraction in response to G protein-linked agonists involves Ca^{++}-calmodulin-dependent activation of MLC kinase and subsequent myosin phosphorylation. More recently, a role for Rho in heterotrimeric GPCR stimulation of blood vessel contraction has been elucidated. This discovery grew out of early observations that in permeabilized blood vessels where Ca^{++} concentration can be maintained at a constant level, contraction could be elicited by the addition of nonhydrolyzable GTP analogs or GTP plus α-adrenergic agonists (Nishimura et al., 1988; Kitazawa et al., 1989). Studies with the C3 exoenzyme revealed that the G protein responsible for this increased responsiveness to Ca^{++} (Ca^{++} sensitization) was the small GTPase Rho (Hirata et al., 1992; Kokubu et al., 1995). Consistent with this finding, Rho translocation in permeabilized blood vessels was induced by GTPγS, AlF_4-, and phenylephrine plus GTP and was quantitatively correlated with Ca^{++} sensitization of contractile force (Gong et al., 1997).

As described above, the Rho effector Rho kinase can regulate MLC phosphorylation. Evidence that this pathway mediates GPCR-stimulated contraction comes from the ability of Y-27632, an inhibitor of Rho kinase, to inhibit Ca^{++} sensitization and vascular contraction in response to a variety of GPCR agonists (Uehata et al., 1997). Thrombin-stimulated contraction of human endothelial cells also was shown to depend on Rho, Rho kinase, and MLC phosphatase (Essler et al., 1998). Additionally, the catalytic subunit of Rho kinase applied to permeabilized vessels results in contraction (Kureishi et al., 1997).

TABLE 1
Evidence for involvement of Rho in GPCR-mediated responses

	Actin Cytoskeletal Rearrangement	FAK Phosphorylation	Cell Migration and Adhesion	Growth and Gene Expression	Contraction/MLC Phosphorylation	Rho Activation
LPA	Ridley, 1992 Jalink, 1994 Tigyi, 1996b Vexler, 1996	Kumagai, 1993 Ridley, 1994	Yoshioka, 1998	Hill, 1995b Mao, 1998	Chrzanowska-Wodnicka, 1996	Fleming, 1996 Aoki, 1998 Gohla, 1998
Thrombin	Jalink, 1994 Majumdar, 1998		Seasholtz, 1999	Majumdar, 1998 Mao, 1998 Seasholtz, 1999	Essler, 1998 Majumdar, 1998	Donovan, 1997 Seasholtz, 1999
PGE_2	Katoh, 1996					
Endothelin	Koyama, 1996	Rankin, 1994 Cazaubon, 1997		Cazaubon, 1997 Kim, 1997	Croxton, 1998	Fleming, 1996
Bombesin	Ridley, 1992	Rankin, 1994				Aoki, 1998
FMLP[a]			Laudanna, 1996			Laudanna, 1996, 1997
Sphingolipids	Sufferlein, 1995 Postma, 1996	Sufferlein, 1995 Wang F, 1997				
Angiotensin α Adrenergic				Aoki, 1998 Sah, 1996 Thorburn, 1997 Hoshijima, 1998	Kokuba, 1995	Aoki, 1998 Gong, 1997 Betuing, 1998
Muscarinic	Togashi, 1998			Fromm, 1997	Croxton, 1998 Kai, 1998	Keller, 1997

Selected or representative publications documenting involvement of Rho in various cellular effects of GPCR agonists are referenced above by the first author's last name and date. The complete list of authors and titles can be found in the reference section.

[a] FMLP, formyl-methionyl-leucyl-phenylalanine.

Rho Involvement in Regulation of Gene Transcription and Cell Growth

A role for Rho in transcriptional regulation of gene expression was first demonstrated in seminal experiments carried out in Treisman's laboratory (Hill et al., 1995b). These studies showed that activated Rho stimulated reporter gene expression regulated by the c-*fos* serum response element (SRE), apparently by enhancing transcriptional activation by serum response factor (SRF). Stimulation of the LPA, endothelin, and m1 muscarinic cholinergic receptors similarly activated the c-fos SRE, and this could be inhibited by C3 exoenzyme (Hill and Treisman 1995a; Bence et al., 1997; Fromm et al., 1997). Very recently, the effects of activated $G_{\alpha 12}$ and $G_{\alpha 13}$, as well as those of Rho exchange factors, on SRE-mediated gene expression have been reported (Fromm et al., 1997; Mao et al., 1998; Fukuhara et al., 1999). The activation of the skeletal α-actin gene by SRF also recently has been shown to be mediated through a Rho-dependent pathway in mouse myoblasts (Wei et al., 1998). There is evidence suggesting that this response is not mediated through Rho kinase, but the Rho effector mediating SRF activation has not been clearly identified (Chihara et al., 1997; Sahai et al., 1998).

The activation of α_1-adrenergic (α_1AdrR) and other G_q-coupled GPCRs in neonatal rat cardiac myocytes leads to transcriptional activation of a number of embryonic and myofilament genes that also are up-regulated during cardiac hypertrophy. These GPCR-mediated responses appear to be dependent on Rho function because dominant negative RhoA and C3 exoenzyme can inhibit responses (Levitzki and Gazit, 1995; Sah et al., 1996; Wang S-M et al., 1997; Aoki et al., 1998; Hoshijima et al., 1998) and GTPase-deficient RhoA can elicit responses like those seen with the agonist (Sah et al., 1996; Aoki et al., 1998; Hoshijima et al., 1998).

Other Rho-Dependent Effects of GPCRs

The function of several enzymes involved in phospholipid metabolism is modulated by Rho. Rho and Arf (another small G protein), along with PKC and the phospholipid phosphatidylinositol biphosphate(PIP_2) and PKC, have been shown to regulate phospholipase D (Brown et al., 1993; Malcolm et al., 1994). Although Rho is clearly involved in GTPγS-mediated phospholipase D activation, a requirement for Rho in GPCR-mediated activation of this enzyme is seen in some, but not all, systems (Malcolm et al., 1996; Mitchell et al., 1998). Another enzyme involved in phospholipid metabolism, phosphatidylinositol 4-phosphate 5-kinase, also has been shown to be regulated by Rho (Chong et al., 1994). Because phosphatidylinositol 4-phosphate 5-kinase activity is necessary for synthesis of the PLC substrate phosphatidylinositol bisphosphate, inactivation of Rho by C3 could inhibit agonist-induced PLC signaling pathways. Indeed, inhibition of Rho function with C3 was shown to prevent thrombin-stimulated Ca^{++} mobilization in mouse fibroblasts (Chong et al., 1994). Another intriguing site of interplay between phospholipid metabolism and Rho function that apparently has not been further explored is the reported association of a RhoGAP (Homma and Emori, 1995) and, more recently, of Rho (Hodson et al., 1998) with the δ isoform of PLC.

An exciting development that recently emerged from Peralta's laboratory (Huang et al., 1993) concerned the involvement of Rho in the control of a delayed rectifying K^+ channel, ($i_{KV1.2}$). These investigators previously demonstrated that mAChR stimulation suppresses this potassium channel through tyrosine phosphorylation. Interestingly, the mAChR effects on $i_{KV1.2}$ appear to be mediated by Rho because C3 toxin inhibits the muscarinic receptor-mediated response and activated RhoA induces tyrosine phosphorylation of $i_{KV1.2}$. Additionally, RhoA was shown by coimmunoprecipitation to directly associate with $i_{KV1.2}$, although it may also regulate channel function indirectly through stimulating a tyrosine kinase (Cachero et al., 1998). Future work may reveal a critical role for Rho in the regulation of other ion channels.

Evidence for Activation of Rho by GPCRs

Studies examining the cellular responses described above have revealed that agonist activation of heterotrimeric G protein-linked receptors can result in signaling to the small G protein Rho. When Rho is activated, e.g., by the addition of GTPγS to cell lysates, and Rho dissociates from the GDI, membrane-associated Rho increases and cytosolic Rho decreases. Thus, changes in the relative cellular distribution of Rho appear to result from and have been used as an indicator of Rho activation. Increases in membrane-associated Rho or decreases in cytosolic Rho have been observed in response to a variety of GPCR agonists. Studies performed on Rat1 fibroblasts showed that LPA increased membrane-associated Rho and decreased cytosolic Rho, as assessed by Western blot analysis (Malcolm et al., 1996). LPA and endothelin also have been demonstrated to increase membrane-associated Rho in intact Swiss 3T3 fibroblasts (Fleming et al., 1996), and angiotensin II, bombesin, and LPA have been shown to increase membrane-associated Rho in intact neonatal cardiomyocytes (Aoki et al., 1998). In permeabilized human embryonic kidney 293 cells, GTPγS decreases cytosolic Rho, and pretreatment of the cells with carbachol enhances this GTPγS-stimulated loss of cytosolic Rho (Keller et al., 1997). The translocation of Rho in response to GTPγS or phenylephrine plus GTP has been associated with Ca^{++} sensitization in α-toxin-permeabilized rabbit portal vein (Gong et al., 1997). At high concentrations (100 U/ml), thrombin was found to increase membrane-associated Rho and decrease cytosolic Rho in primary rat astrocytes (Donovan et al., 1997). Finally, we have shown that low concentrations of thrombin (0.5 U/ml) increase levels of membrane-associated Rho in intact rat aortic smooth muscle cells (Seasholtz et al., 1999) and enhance GTPγS-stimulated Rho redistribution in astrocytoma cell lysates (T. Seasholtz, unpublished observation).

Direct evidence for GPCR-mediated activation of Rho based on an increase in the fraction of Rho in the GTP-liganded state is more limited. The high rate of GTP hydrolysis by Rho makes agonist-induced increases in ^{32}P-GTP difficult to detect in Rho immunoprecipitates, but increases in ^{32}P-GDP or nonhydrolyzable [^{35}S]GTPγS on Rho have been observed after treatment of leukocytes with the chemoattractant formyl-methionyl-leucyl-phenylalanine or interleukin-8 (Laudanna et al., 1996, 1997). More recently, stimulation of preadipocytes with α_2AdrR agonists was shown to increase ^{32}P-GTP and decrease ^{32}P-GDP in Rho immunoprecipitates (Betuing et al., 1998). Our laboratory also has shown that thrombin and the thrombin peptide SFLLRNP stimulate Rho-[^{35}S]GTPγS binding in lysates of

primary rat aortic smooth muscle cells (Seasholtz et al., 1999) and 1321N1 astrocytoma cells (T. Seasholtz, unpublished observation). Activated α subunits of G_{12} or G_{13} also increase the amount of ^{32}P-GTP-associated RhoA in ^{32}P-orthophosphate-labeled COS-7 cells (Gohla et al., 1998), providing direct evidence for functional coupling between heterotrimeric and small G proteins. Although the magnitude of the increases in Rho-GTP binding or redistribution are usually less than 2-fold, this is not dissimilar to the magnitude of increases in activated Ras generally observed in response to GPCR stimulation. A newly developed assay to measure GTP-bound Rho, as assessed by affinity-precipitation of Rho by the Rho binding domain of its effector, rhotekin, demonstrated an almost 3-fold stimulation by LPA in Swiss 3T3 fibroblasts (Ren et al., 1999).

Identification of G Proteins Activating Rho

The most intriguing question that remains to be answered is how GPCRs signal to and activate Rho. Both the nature of the G protein subunits that mediate this response and the molecular mechanisms involved are under intensive study. LPA-induced increases in membrane-associated Rho were reported to be pertussis toxin sensitive, suggesting that a member of the G_i or G_o family might be involved (Fleming et al., 1996). In preadipocytes, α_2AdrR activation of Rho also is pertussis toxin sensitive (Betuing et al., 1998), suggesting that $G_{\alpha i/o}$ may activate Rho in some systems. In contrast, the majority of GPCR-induced, Rho-mediated effects on the cytoskeleton are pertussis toxin insensitive (Jalink and Moolenaar, 1992; Ridley and Hall, 1994; Tigyi et al., 1996a; Majumdar et al., 1998), and constitutively activated $G_{\alpha i}$ was not observed to induce cell rounding (Katoh et al., 1998) or stress fiber formation (Buhl et al., 1995).

Several lines of recent evidence suggest that G proteins of the pertussis toxin–insensitive $G_{12/13}$ family control Rho-dependent stress fiber formation. Johnson's laboratory (Buhl et al., 1995) was the first to show that the microinjection of either $G_{\alpha 12}$ or $G_{\alpha 13}$ into Swiss 3T3 fibroblasts resulted in stress fiber formation, a response which was blocked by pretreatment with the C3 exoenzyme. Barber's laboratory (Hooley et al., 1996) also demonstrated that a GTPase-deficient, activated mutant of $G_{\alpha 13}$ produces stress fibers and activates the Na^+/H^+ exchanger isoform NHE1 through a Rho-dependent pathway in CCL39 fibroblasts. Interestingly, in this system, $G_{\alpha 12}$ was found to inhibit Na^+/H^+ exchange by NHE1 (Lin et al., 1996). A more recent study confirmed that the microinjection of either activated $G_{\alpha 12}$ or $G_{\alpha 13}$ into Swiss 3T3 cells resulted in Rho-dependent production of actin stress fibers and focal adhesions. However, only antibodies to $G_{\alpha 13}$ were able to block the LPA-mediated cytoskeletal organization, indicating that LPA signals through G_{13} to produce this Rho-mediated effect (Gohla et al., 1998). In contrast, we find that thrombin-induced cell rounding is blocked by antibodies to $G_{\alpha 12}$ (M.M., C. Buckmaster, D. Toksoz, T.M.S., and J.H.B., in preparation). Experiments with inhibitory forms of $G_{\alpha 12}$ and $G_{\alpha 13}$ also suggest that thrombin elicits its effects on stress fiber formation via G_{12} and LPA via G_{13} (A. Gohla et al., personal communication). Thus, GPCR agonists may use several distinct G proteins and signaling pathways to elicit Rho activation and mediate cytoskeletal change. In contrast, in mouse platelets, activation of both $G_{\alpha 12}$ and $G_{\alpha 13}$ has been demonstrated in response to stimulation with thromboxane A_2 or thrombin (Klages et al., 1999). Stimulation of $G_{\alpha 12/13}$-dependent MLC phosphorylation and platelet shape change by thromboxane A_2 receptors in $G_{\alpha q}^{-/-}$ cells were shown to be dependent on both Rho and Rho kinase (Klages et al., 1999), indicating that this receptor potentially signals through both family members to elicit Rho-dependent effects. $G_{\alpha 12}$ and $G_{\alpha 13}$ also have been shown to stimulate Rho-dependent tyrosine phosphorylation of focal adhesion kinase, paxillin, and $p130^{cas}$, as do the agonists LPA or bombesin (Needham and Rozengurt, 1998).

Cell rounding and neurite retraction also are mediated through $G_{\alpha 12}$- and $G_{\alpha 13}$-controlled pathways. In our studies on 1321N1 astrocytoma cells, the microinjection of the expression plasmids for either $G_{\alpha 12}$ or $G_{\alpha 13}$ mimicked the previously reported effects of thrombin, including the retraction of processes and cell rounding (M. Majumdar, C. Buckmaster, D. Toksoz, T. Seasholtz, and J. H. Brown, in preparation). The effects of $G_{\alpha 12}$ and thrombin were inhibited not only by the microinjection of the C3 exoenzyme, but also by the microinjection of cDNA encoding for a DH deletion mutant of the Rho exchange factor lbc (M. Majumdar, C. Buckmaster, D. Toksoz, T. Seasholtz, and J. H. Brown, in preparation). Studies recently reported by Katoh et al. (1998) demonstrated that activated $G_{\alpha q}$, $G_{\alpha 12}$, and $G_{\alpha 13}$ all induce Rho-dependent neurite retraction and cell rounding, but via different mechanisms. The tyrosine kinase inhibitor tyrphostin A25 blocked morphological changes mediated by both $G_{\alpha q}$ and $G_{\alpha 13}$, but not those induced by $G_{\alpha 12}$. In contrast, inhibition of PKC or the elimination of intracellular Ca^{++} blocked responses to $G_{\alpha q}$, but not to $G_{\alpha 12}$ or $G_{\alpha 13}$. Both tyrphostin A25 and the epidermal growth factor receptor-specific compound AG1478 also were shown by Gohla et al. (1998) to block stress fibers in response to LPA or activated $G_{\alpha 13}$, but not in response to activated $G_{\alpha 12}$. This is consistent with earlier observations by Nobes et al. (1995) demonstrating tyrosine kinase involvement in activation of stress fibers by LPA, but not in response to activated RhoA, indicating that tyrosine kinases may be involved in the pathway from GPCRs to Rho activation. These data further suggest that there are multiple GPCR/G protein-specific pathways for Rho activation.

GPCRs As Regulators of RhoGEFs

A series of pivotal papers from the Sternweis and Hart laboratories (Kozasa et al., 1998; Hart et al., 1998) have provided direct evidence for a mechanism by which heterotrimeric G proteins of the G_{12}/G_{13} family can activate Rho. These studies revealed that the p115-RhoGEF contains a regulator of G protein signaling-like domain and acts as a GAP for both $G_{\alpha 12}$ and $G_{\alpha 13}$ (Kozasa et al., 1998; Hart et al., 1998). Previous studies have shown that the $G_{\alpha q}$ effector PLC can act as a GAP for $G_{\alpha q}$ and that by analogy, p115-RhoGEF would appear to be a possible effector of $G_{\alpha 12}$ and/or $G_{\alpha 13}$. Indeed, additional experiments revealed that purified $G_{\alpha 13}$ was able to stimulate the Rho exchange activity of p115-RhoGEF (as assessed by dissociation of GDP from Rho). These findings provide the first evidence of an interaction of G_α subunits of the $G_{12/13}$ family with a Rho-specific GEF and go on to define the p115-RhoGEF as the putative effector of $G_{\alpha 13}$ signaling. Interestingly, although p115-RhoGEF was shown to serve as a GAP for $G_{\alpha 12}$ as well as $G_{\alpha 13}$, an increase

in the rate of p115-RhoGEF-catalyzed guanine nucleotide exchange on Rho was not stimulated by $G_{\alpha 12}$. Subsequently, Mao et al. (1998) demonstrated that $G_{\alpha 13}$ synergizes with p115-RhoGEF to activate SRF-mediated gene expression, whereas $G_{\alpha 12}$ does not. The findings that $G_{\alpha 12}$ does not enhance the nucleotide exchange of Rho by p115-RhoGEF or synergize in SRE-mediated gene transcription suggest that this α subunit may induce Rho activation through a different GEF. By searching DNA databases with DH domain consensus sequences, Gutkind's laboratory (Fukuhara et al., 1999) recently identified another putative RhoGEF, first described as KIAA380, that contains a PDZ domain that has been termed PDZ-RhoGEF. The PDZ-RhoGEF also was shown to directly associate with both $G_{\alpha 12}$ and $G_{\alpha 13}$, although neither its activation nor its ability to serve as a GAP was examined in this report.

Although it is intriguing to consider that a heterotrimeric G protein α subunit such as $G_{\alpha 12}$ and/or $G_{\alpha 13}$ interacts directly with and, thus, activates a Rho exchange factor, additional regulatory pathways for control of Rho activation are likely. The GPCRs that have been shown to couple to $G_{\alpha 12/13}$ appear to be those that also couple to $G_{\alpha q}$ (Offermanns et al., 1994; Barr et al., 1997). Likewise, the majority of GPCRs shown to induce redistribution of Rho are known to link to $G_{\alpha q}$ (and in some cases, to $G_{\alpha 12/13}$). Because coupling to G_q leads to activation of PKC, it is likely that this kinase might regulate Rho function. The possibility that PKC might phosphorylate and regulate Rho exchange factor(s) or RhoGAPs is suggested by the finding that PKC phosphorylation of the RacGEF Tiam has been reported (Fleming et al., 1997). PKC also has been shown to phosphorylate $G_{\alpha 12}$ and $G_{\alpha 13}$ (Kozasa and Gilman, 1996; Offermanns et al., 1996), providing the possibility of an additional level of $G_{\alpha q}$ regulation of Rho signaling. Furthermore, in light of the apparent involvement of tyrosine kinases in the G protein-induced cytoskeletal responses described above, tyrosine phosphorylation of GEFs or other regulatory proteins may also contribute to Rho activation.

A signaling role of $\beta\gamma$ subunits of heterotrimeric G proteins is well documented and may be the predominant pathway for transducing certain G_i-mediated responses. Bovine brain $G_{\beta\gamma}$ was shown to bind to Rho and inhibit Rho-GTPγS binding (Harhammer et al., 1996). Although the functional significance of this interaction is unknown, the authors speculate that $G_{\beta\gamma}$ may target Rho to the membrane and/or possess RhoGAP activity. Because $G_{\beta\gamma}$ subunits used in the above-mentioned studies were isolated from $G_{i/o}$, it is conceivable that, in some cases, pertussis toxin-sensitive regulation of Rho could be mediated through $G_{\beta\gamma}$. Rho activation could occur through effects of $\beta\gamma$ subunits on protein kinase cascades, as described for the regulation of Ras activation by $\beta\gamma$.

There also is accumulating evidence for the regulation of Rho-dependent pathways through cyclic AMP (cAMP), and, thus, conceivably through $G_{\alpha s}$. The mechanism(s) underlying the inhibitory effect of cAMP on Rho is not fully understood, but cAMP or protein kinase A (PKA) may act at several sites. One report demonstrated that PKA-dependent phosphorylation of Rho was associated with increases in cytosolic Rho, although changes in guanine nucleotide binding were not seen (Lang et al., 1996). Another group showed that agonist-stimulated [^{35}S]GTPγS binding to Rho was inhibited by 8-bromo-cAMP, a cAMP analog (Laudanna et al., 1997). Further evidence for PKA-dependent inhibition of Rho function was presented in a recent study (Dong et al., 1998), demonstrating that morphological responses to cAMP observed in several neuronal cell lines were abolished by the expression of a mutant form of RhoA that was not a substrate for PKA (Dong et al., 1998). Of additional interest, in two recent reports, cAMP was shown to directly bind to and activate a GEF for Rap1A (another small GTPase), independent of PKA (de Rooij et al., 1998; Kawasaki et al., 1998). These observations suggest the possibility that GPCRs linked to cAMP formation could also regulate RhoGEF activity.

Summary

The regulation and functions of large and small G proteins have long been studied independently. It is now evident that Rho and other small G proteins of the Rho family can be activated through the stimulation of heterotrimeric G proteins, blurring the boundaries between these signaling systems. Although the ability of specific G α subunits to directly activate GEFs may be unique to the pathway linking $G_{12/13}$ to Rho, it seems more likely that mechanisms such as these will be conserved. Further discoveries of such interactions may reveal additional novel pathways through which GPCR activation can elicit responses as diverse as contraction, cytokinesis, cell motility, and transformation.

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