A Rho Exchange Factor Mediates Thrombin and Ga12-induced Cytoskeletal Responses*

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Thrombin induces astrocytoma cell rounding through a Rho-dependent pathway (Majumdar, M., Seasholtz, T. M., Goldstein, D., de Lanerolle, P., and Brown, J. H. (1998) J. Biol. Chem. 273, 10099–10106). The involvement of the Ga12 family of G proteins and the role of specific Rho exchange factors in transducing signals from the thrombin receptor to Rho-dependent cytoskeletal responses was examined. Microinjection of cDNAs for activated Ga12 or Ga13 induced cell rounding, and antibodies to Ga12 or Ga13 blocked the response to thrombin. In contrast, activation or inhibition of Gaq function had relatively little effect. The cytoskeletal response to Ga12 was inhibited by microinjection of C3 exoenzyme, indicating Rho dependence. Two Rho-specific guanine nucleotide exchange factors (GEFs), oncogenic lbc and p115, increased the percentage of rounded cells 4–5-fold, and this was inhibited by C3. Mutant GEFs lacking the Dbl homology (DH) domain required for exchange factor activity failed to induce cell rounding. However, the DH mutants of lbc and p115 were efficacious inhibitors of rounding induced by thrombin or Ga12. The effects of lbc were dependent on an intact pleckstrin homology domain, which may be required for appropriate targeting of the Rho-GEF. These findings identify the Ga12 protein family as transducers of thrombin signaling to the cytoskeleton and provide the first evidence that a Rho-GEF transduces signals between G protein-coupled receptors and Rho-mediated cytoskeletal responses.

The small G protein Rho is one of a family of low molecular weight GTPases that act as molecular switches to regulate cellular responses. Among its many functions, Rho is involved in controlling the actin cytoskeleton, thereby regulating cell shape and polarity, cytokinesis, cell motility, and contraction (1, 2). Ligands for G protein-coupled receptors, including lysophosphatidic acid (LPA),1 bombesin, thrombin, and endothelin, have been shown to induce actin cytoskeletal rearrangements in a variety of cell types (3–7). These GPCR agonist-induced morphological changes are mimicked by microinjection of activated Rho and are abolished by C3 exoenzyme pretreatment (3–5), which specifically ribosylates and inhibits Rho function.

In neuronal cell lines and PC-12 cells, LPA, thrombin, and progaglandin E2 (all ligands for GPCRs) induce Rho-dependent retraction of cell processes and cell rounding (4, 6, 8). Our laboratory has also demonstrated a requirement for Rho in thrombin-stimulated rounding and retraction of processes in 1321N1 astrocytoma cells (9). Similar cytoskeletal rearrangements are believed to be crucial in growth cone guidance and cell migration and may be involved in development and plasticity of neuronal and glial cells.

Despite abundant evidence for the involvement of Rho family small G proteins in agonist-induced morphological responses, there is limited information regarding the molecular mechanisms linking agonist activation of GPCR to regulation of Rho-mediated events. The question of which heterotrimeric G proteins are responsible for coupling receptors to the downstream targets that transduce the effects of agonists on the actin cytoskeleton is of considerable interest. Notably, GPCR agonists that activate phospholipase C through Gaq are not equally efficacious for inducing cytoskeletal changes. For example, thrombin but not carbacbol causes cell rounding in astrocytoma cells (9), and LPA but not bradykinin causes process retraction in PC-12 cells (6). Several lines of evidence support the conclusion that thrombin-induced cell rounding is independent of phospholipase C activation, Ca2+ mobilization, or pertussis toxin-sensitive G protein activation (9, 10). Thus, heterotrimeric G proteins other than those of the Gaq or Ga12 family are likely to be responsible for linking activation of selected GPCRs to Rho-mediated actin rearrangements.

The pertussis toxin-insensitive G protein Ga13 was first isolated as an oncogene (11) and subsequently shown to induce cellular transformation in fibroblasts (12, 13). Previous work from our laboratory used microinjection of antibodies to the C terminus of Ga subunits to demonstrate that the thrombin receptor regulates DNA synthesis in 1321N1 cells through Ga12 (14). Other recently identified cellular functions that are regulated by Ga12 or its closely related family member Ga13 include stress fiber formation in fibroblasts (15, 16), SRE-mediated gene transcription (12, 17), activation of JNK (18–20), and activation of Na+/H+ exchange (21, 22). However, the direct effectors of Ga12/13 that mediate the aforementioned responses still remain to be identified.

Activation of Rho and other small GTPases requires the exchange of GDP for GTP, a process catalyzed by guanine nucleotide exchange factors (GEFs). Several GEFs including lbc, lfc, lsc, and p115 show specificity for Rho (23, 24). Very recent studies provide evidence that the Rho-specific GEFs can transduce signals from the Ga12 family of heterotrimeric G proteins to Rho. The p115 GEF was demonstrated to contain an RGS-like domain and act as a GTPase-activating protein for Ga12 and Ga13 (25, 26). Most importantly, Ga13 was demonstrated to enhance the Rho exchange activity of p115 (25). A

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‡ The abbreviations used are: LPA, lysophosphatidic acid; GEF, guanine nucleotide exchange factor; DMEM, Dulbecco's modified Eagle's medium; PH, pleckstrin homology; SRE, serum response element; GPCR, G protein-coupled receptor; DH, Dbl homology domain.

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related observation is that G\textsubscript{12} can synergize with p115 to activate SRE-mediated gene transcription (17). Another G\textsubscript{12} family binding protein, termed PDZ-RhoGEF or KIAA0380, also regulates G\textsubscript{12} effects on SRE activation (27). Involvement of Rho exchange factors in GPCR- or G\textsubscript{12,13}-mediated effects on the actin cytoskeleton has not been demonstrated.

We hypothesized that the thrombin receptor mediates cell rounding through coupling to G\textsubscript{12}, and subsequent activation of a Rho exchange factor. To test this possibility we microinjected inhibitory antibody to G\textsubscript{12}, as well as an expression plasmid for activated G\textsubscript{12}. Our findings indicate that G\textsubscript{12} is necessary and sufficient for thrombin-induced cell rounding.

Furthermore, we demonstrate that thrombin- and G\textsubscript{12,13}-stimulated rounding are inhibited by mutants of the Rho-GEFs Lbc and p115. These data implicate GEFs as downstream mediators of G\textsubscript{12,13}-induced Rho-dependent cytoskeletal changes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Human 1321N1 astrocytoma cells were plated onto 100-mm plates at a density of 1.2 x 10\textsuperscript{6} cells/ml and grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, (100 \mu g/ml) for 4 days. The cells were then tritured and set on 12-mm round glass coverslips (preshewed with acid and ethanol) at a density of ~1 x 10\textsuperscript{4} cells/slip and allowed to grow in 5% fetal calf serum plus DMEM for 48 h before microinjection.

**C3 Toxin Fusion Protein—**CDNA for the glutathione S-transferase-C3 fusion protein (kindly provided by Dr. J. Meinkoth, University of Pennsylvania) was used to transform JM109 Escherichia coli to produce the glutathione S-transferase-C3 fusion protein for purification. After transformation, the cells were lysed, and clarified extracts were incubated with GSH-Sepharose. After extensive washing, the C3 toxin protein was cleaved from the glutathione S-transferase by overnight incubation with thrombin. Thrombin was removed by incubation with para-aminobenzamidine-Sepharose, and the supernatant was concentrated in a Centricon-10 to a final concentration of 5 mg/ml protein. In experiments involving microinjection of the C3 toxin, the protein was first diluted to a concentration of 40 \mu g/ml in microinjection buffer (100 mM KC1, 5 mM NaPO\textsubscript{4}) before injection.

**Microinjection—**1321N1 cells were set on glass coverslips and grown as described above (see “Cell Culture”). Expression plasmids were prepared by CsCl gradient and diluted to a concentration of 50 \mu g/ml in microinjection buffer. These diluted expression plasmids were microinjected into cell nuclei along with 100 \mu g/ml of a marker plasmid for nuclear green fluorescent protein (provided by the laboratory of Dr. Geoff Wahl (28)) using an Eppendorf microinjector and Zeiss Axiovert Microscope. After microinjection of expression plasmids, cells were incubated in DMEM with 5% fetal calf serum for 3 h, followed by 20 min in serum-free DMEM plus 0.1% bovine serum albumin with or without thrombin (0.5 units/ml), and then fixed and stained as described below. For microinjection of antibodies, the antibodies were concentrated to 10 mg/ml using a Centricon and then diluted to 5 mg/ml with IgG. Following microinjection, cells were incubated for 30 min in 5% DMEM plus fetal calf serum, after which they were switched to serum-free medium plus 0.1% bovine serum albumin with or without thrombin (0.5 units/ml) for 20 min. Cells were then fixed and stained for F-actin as described below. Microinjected cells were identified by staining for co-injected IgG or by directly staining for the injected antibody (from rabbit) using a secondary anti-rabbit antibody conjugated to fluorescein isothiocyanate.

**Rounding (Reversal of Stellation) Assay and Immunofluorescence—**To detect actin morphology of microinjected cells, cells were fixed in 3.7% formaldehyde/phosphate-buffered saline, permeabilized with 0.3% Triton X-100/phosphate-buffered saline, and stained for 30 min with rhodamine-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR). Microinjected cells were identified by detection of green fluorescent nuclei (expressing co-injected nuclear green fluorescent protein), and F-actin staining was visualized using a Zeiss-Axioskop microscope and a x 40 Neofluar objective lens. Approximately 100–150 injected cells were detected per slip, and these were scored for rounding based on absence of “stellate” morphology (i.e. lack of processes and rounded morphology).

**Statistical Analysis—**Data were analyzed by analysis of variance. Postanalysis was performed using the Tukey test when p was <0.05. p values are given in the figure legends.

**RESULTS**

**Microinjected Antibodies to G\textsubscript{12} and G\textsubscript{13} Inhibit Thrombin-induced 1321N1 Cell Rounding—**To determine whether G\textsubscript{12} mediates the cytoskeletal effects of thrombin we microinjected a G\textsubscript{12} C-terminal antibody into 1321N1 cells. The ability of thrombin to induce cell rounding was examined 30 min after antibody injection and compared with the response in cells microinjected with IgG. The G\textsubscript{12} C-terminal antibody inhibited thrombin-induced rounding by nearly 80% (Fig. 1). Microinjection of a G\textsubscript{13} antibody (CT100 from Dr. Melvin Simon) also dramatically decreased rounding. We also tested a commercially available C-terminal G\textsubscript{13} antibody (Calbiochem) which gave a marked but less complete (~55%) inhibition (data not shown). The same concentration of a C-terminal G\textsubscript{12} antibody caused only partial inhibition (~30%) of thrombin-induced rounding. To determine the specificity of the G\textsubscript{12} and G\textsubscript{13} antibodies, Western blot analysis was performed on lysates from COS cells transfected with plasmids encoding G\textsubscript{12} or G\textsubscript{13}, as described previously (14). The G\textsubscript{12} antibody recognized only G\textsubscript{12}; however, both G\textsubscript{13} antibodies recognized G\textsubscript{13} and, to a lesser extent, G\textsubscript{12} (data not shown). Therefore, the ability of the G\textsubscript{13} antibody to inhibit thrombin-stimulated rounding may be due in part to blockade of G\textsubscript{12} function.

**Constitutively Activated G\textsubscript{12} and G\textsubscript{13} Induce Cell Rounding—**To determine which Go subunits could mimic the effects of thrombin, 1321N1 cells were microinjected with 50 \mu g/ml expression plasmids for the activated form of Go\textsubscript{q} (Go\textsubscript{qRC}), G\textsubscript{12} (G\textsubscript{12QL}), or Go\textsubscript{13} (Go\textsubscript{13QL}). The cells were fixed 3 h after injection and stained, and the percentage of round cells was determined. Approximately 10% of cells injected with control plasmid (pCIS) were rounded (Fig. 2A), while most showed a normal stellate, process-bearing shape like that seen in uninjected cells (Fig. 2B). Microinjection of constitutively activated G\textsubscript{12} or G\textsubscript{13} expression plasmids induced a loss of stellation and produced a round morphology in 40–50% of the injected cells. The morphology induced by microinjection of activated G\textsubscript{12QL} or G\textsubscript{13QL} was identical to that seen with thrombin stimulation of astrocytoma cells (9). Microinjection of the same concentration of constitutively activated Go\textsubscript{q} cDNA resulted in a significantly lower level of cell rounding (~20% of injected cells) than that seen with activated G\textsubscript{12} or G\textsubscript{13}. Activated Go\textsubscript{q} was less efficacious than G\textsubscript{12} even when the concentration of microinjected Go\textsubscript{q} was 50-fold above that of G\textsubscript{12} (data not shown).

**Activated Rho, but Not Activated Ras or Activated Rac, Induces 1321N1 Cell Rounding—**A variety of cell-specific cytoskeletal proteins have been shown to mediate cell rounding in response to various stimuli (9). RhoA is known to associate with membrane elements (9), and stimulation of astrocytoma cells (9). Microinjection of the same concentration of constitutively activated Go\textsubscript{q} cDNA resulted in a significantly lower level of cell rounding (~20% of injected cells) than that seen with activated G\textsubscript{12} or G\textsubscript{13}. Activated Go\textsubscript{q} was less efficacious than G\textsubscript{12} even when the concentration of microinjected Go\textsubscript{q} was 50-fold above that of G\textsubscript{12} (data not shown).

**FIG. 1.** Microinjection of antibodies to heterotrimeric G protein \( \alpha \) subunits inhibits thrombin-induced cell rounding. Cells were microinjected with antibodies against the C-terminal domains of the \( \alpha \) subunits for Go\textsubscript{q}, G\textsubscript{12}, and G\textsubscript{13} at a final concentration of 5 mg/ml. Thirty minutes after injection, cells were changed to serum-free medium plus 0.1% bovine serum albumin with or without thrombin (0.5 units/ml) for 20 min and then fixed and stained for F-actin as described under “Experimental Procedures.” Data are expressed as the mean ± S.E. from two separate experiments, each containing two or three coverslips. *, \( p < 0.05 \) compared with IgG plus thrombin; **, \( p < 0.001 \) compared with IgG plus thrombin.
toskeletal responses have been shown to be induced by small G proteins. To directly examine the effect of Rho on astrocytoma cell morphology, we microinjected an expression plasmid for constitutively activated Rho (L63Rho), activated Rac (V12Rac), or activated Ras (V12Ras). Microinjected cells were identified and scored for rounding as described under "Experimental Procedures." The results are expressed as the means ± S.E. from either two experiments, each containing one to two coverslips (for vector and L63Rho), or five experiments, each containing two or three coverslips (for V12Rac and V12Ras), for activated Rac (V12Rac) or activated Ras (V12Ras). Microinjection of activated Gα12 stimulates cell rounding through a Rho-dependent mechanism, we coinjected C3 exoenzyme protein along with expression plasmids for vector, constitutively activated Rho (L63Rho), activated Rac (V12Rac), or activated Ras (V12Ras). Microinjected cells were identified and scored for rounding as described under "Experimental Procedures." The results are expressed as the means ± S.E. from either two experiments, each containing one to two coverslips (for V12Rac and V12Ras) or five experiments, each containing two or three coverslips (for vector and L63Rho). **, p < 0.001 compared with control vector (pCIS); ***, p < 0.001 compared with control vector (pCMV5).

Activation of Rho-dependent Cell Rounding by a Rho-GEF—To determine whether Rho-specific guanine nucleotide exchange factors, which serve as activators of Rho, could effect rounding in 1321N1 cells.2

Activation of Rho-dependent Cell Rounding by a Rho-GEF—To determine whether Rho-specific guanine nucleotide exchange factors, which serve as activators of Rho, could effect similar changes in 1321N1 cell shape, we microinjected expression plasmids for the Rho-specific GEF, lbc (see Fig. 5A). A truncated, activated form of lbc (onco-lbc), which lacks the

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2 M. Majumdar, D. Goldstein, and J. Heller Brown, unpublished observations.
region C-terminal to but includes the pleckstrin homology (PH) domain was found to be a highly efficacious activator of cell rounding (Fig. 6A). As evidence that this response was mediated through the ability of lbc to activate Rho, we demonstrated that onco-lbc-induced cell rounding was inhibited by C3 co-injection (Fig. 6A). Microinjection of the full-length proto-lbc construct had a significant but lesser effect on cell morphology. Expression plasmids for another Rho-specific exchange factor, p115 (Fig. 5B), were also microinjected. Both the full-length form (p115) and an N-terminal, truncated cDNA (ΔN-p115), which lacks the region that contains RGS sequence homology (26), induced cell rounding (Fig. 6B). The response to p115 was shown to be Rho-dependent based on inhibition by C3 exoenzyme co-injection (Fig. 6B).

The Rho-GEF Mutants Lacking Dbl Homology (DH) Domains Are Inactive but Inhibit Thrombin- and G12-stimulated Cell Rounding—Mutants of lbc or p115 lacking DH domains cannot catalyze GDP/GTP exchange (24, 31). To investigate a role for lbc and/or p115 in the signaling pathway utilized by thrombin and G12, we examined the possibility that DH domain mutants of the Rho-GEFs would act to inhibit downstream signaling. Mutants of lbc and p115 lacking their DH domains were first examined for their ability to induce cell rounding (Fig. 7, A and B). In contrast to the marked stimulatory effects of onco-lbc or p115 shown in Fig. 6, A or B, the comparable mutants lacking DH domains (“lbc no DH” or “p115 no DH”; see Fig. 5) were ineffective (Fig. 7, A and B). Of greatest interest, expression of lbc no DH or p115 no DH inhibited cell rounding induced by thrombin by more than 80% (Fig. 7, A and B). Additionally, injection of the mutants lbc no DH or p115 no DH caused nearly complete inhibition of the induction of cell rounding by co-injected G12 (Fig. 7, A and B). As evidence that inhibition by the no DH mutant occurred upstream of Rho, lbc no DH failed to significantly inhibit cell rounding induced by activated (L63)Rho. To control for the specificity of the inhibition, we further determined that lbc no DH did not block cell rounding induced by colchicine (data not shown), which presumably elicits cytoskeletal responses through its direct effect on microtubules. An lbc mutant lacking the PH domain had no significant effect on cell morphology but, unlike the no DH mutant, did not act as an inhibitor of thrombin signaling (Table I).

DISCUSSION

The thrombin receptor has been suggested to couple to G12 and G13 based on its ability to increase GTP binding to these PTX-insensitive heterotrimeric G proteins in platelets and a reconstituted baculovirus system (32, 33). Our laboratory has also shown G12 to be required for thrombin-stimulated mitogenesis, Ras activation, and AP-1-mediated gene transcription (14, 20, 34). However, because these transcriptional and mitogenic responses result from convergence of multiple pathways and are measured at long times following thrombin stimulation, it has been difficult to identify the effectors immediately downstream of thrombin and G12 signaling in these pathways. The study described here presents evidence that thrombin-induced cytoskeletal rearrangement in 1321N1 astrocytoma cells is also mediated through receptor coupling to the heterotrimeric G protein G12. Analysis of this rapid and robust response has allowed further dissection of the molecular events by which G12 signaling occurs (Fig. 8).

Several observations support the involvement of G12 in thrombin-mediated cytoskeletal rearrangement. First, microinjection of the constitutively activated subunit of G12, alone, in the absence of agonist, is sufficient to elicit a morphological response (i.e. cell rounding and retraction of processes) identical to that seen with thrombin treatment. This finding is consistent with a recent study, published while this manuscript was in preparation, which found retraction of neurites in PC-12 cells to be induced by activated G12 (35). More compelling evidence supporting the involvement of G12 in thrombin receptor-induced 1321N1 cell rounding is the observation that the response is fully inhibited by microinjection of C-terminal inhibitory antibodies to G13. The finding that thrombin-induced rounding could also be blocked by microinjection of an antibody to the C terminus of G13 suggested that the G12
and G13 pathways might both be required for thrombin-induced rounding. However, while the Go12 antibody was specific for Go12, both Go13 antibodies tested reacted with Go12 as well as Go13. Thus, the inhibitory effect of the Go13 antibodies may result from blockade of Go12 signaling. Gohlke et al. (16) examined the ability of antibodies to the C-terminal of Go12 and Go13 to block LPA-induced stress fiber formation and reported inhibition only with antibodies to Go13. More recent work from the same group has shown that antibodies to Go12, but not Go13, were capable of inhibiting thrombin-stimulated stress fiber formation (42). These findings suggest that the LPA receptor preferentially couples to Go13, while the thrombin receptor couples to Go12 to induce cytoskeletal responses.

Our findings further implicate a Rho guanine nucleotide exchange factor in the thrombin receptor/Go12/13-induced cytoskeletal response. Specifically, we demonstrate that two distinct Rho exchange factors, lbc and p115, both stimulate cell rounding and retraction of cell processes. The response to these Rho-GEFs, as well as the response to thrombin and Go12, is C3-sensitive, demonstrating that Rho mediates the effects of all of these stimuli. Further experiments show that the guanine nucleotide exchange activity, which is localized to the DH domain, is required for this Rho-GEF-induced cytoskeletal response, since neither the lbc nor p115 mutants which lack exchange activity stimulate rounding. Microinjection of a mutant lbc, which lacks the PH domain, was also unable to induce cell rounding or process retraction, demonstrating a requirement for the PH domain in these morphological responses. Surprisingly, this same mutant was able to induce stress fibers in fibroblasts (31). However, the PH domain has been shown to be required for transformation (24, 31, 36–38), has a critical role in targeting GEFs to their site of action (31, 39) and appears to be important for optimal guanine nucleotide exchange activity (38, 40).

Most critical to the conclusion that Rho-GEFs transduce signals from thrombin/Go12 to Rho is the finding that microinjection of either lbc or p115 mutants lacking the DH exchange factor domain (lbc no DH or p115 no DH) can inhibit thrombin- and Go12-stimulated rounding. Importantly, the lbc no DH
Notably, these studies reported that a tyrphostin A25-sensitive tyrosine kinase was required for the effects of LPA and Go12 on Rho-dependent cytoskeletal responses (16, 35, 41) but that the response to Go12 was not blocked by this tyrosine kinase inhibitor (16, 35). Our preliminary data also suggest that the thrombin/Go12-induced morphological response is not inhibited by tyrphostin A25 or PP2, which inhibits src-like tyrosine kinases. However, studies using more selective inhibitors of Src family or other nonreceptor tyrosine kinases are required to rule out involvement of tyrosine kinases in the pathway by which thrombin and Go12 activate Rho-GEFs and elicit cell rounding.

The studies reported here are among the first to show that mutant GEFs can be used to inhibit signaling through Rho-dependent pathways and to demonstrate that a mutant Rho-GEF can block agonist or heterotrimeric G protein-stimulated cytoskeletal responses. Other recent studies demonstrate inhibition of agonist and Go12-induced effects on SRE-mediated gene expression by no DH mutants of p115 and PDZ-RhoGEF (17, 27). We are currently investigating the mechanism and specificities of Go12 signaling to Rho exchange factors and the question of how Rho is activated in response to GPCR agonists and heterotrimeric G proteins.

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