Regulation of G Protein-linked Guanine Nucleotide Exchange Factors for Rho, PDZ-RhoGEF, and LARG by Tyrosine Phosphorylation

EVIDENCE OF A ROLE FOR FOCAL ADHESION KINASE*

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A recently identified family of guanine nucleotide exchange factors for Rho that includes PDZ-RhoGEF, LARG, and p115RhoGEF exhibits a unique structural feature consisting in the presence of area of similarity to regulators of G protein signaling (RGS). This RGS-like (RGL) domain provides a structural motif by which heterotrimeric G protein α subunits of the Go12 family can bind and regulate the activity of RhoGEFs. Hence, these newly discovered RGL domain-containing RhoGEFs provide a direct link from Go12 and Go13 to Rho. Recently available data suggest, however, that tyrosine kinases can regulate the ability of G protein-coupled receptors (GPCRs) to stimulate Rho, although the underlying molecular mechanisms are still unknown. Here, we found that the activation of thrombin receptors endogenously expressed in HEK-293T cells leads to a remarkable increase in the levels of GTP-bound Rho within 1 min (11-fold) and a more limited but sustained activation (4-fold) thereafter, which lasts even for several hours. Interestingly, tyrosine kinase inhibitors did not affect the early phase of Rho activation, immediately after thrombin addition, but diminished the levels of GTP-bound Rho during the delayed phase. As thrombin receptors stimulate focal adhesion kinase (FAK) potently, we explored whether this non-receptor tyrosine kinase participates in the activation of Rho by GPCRs. We obtained evidence that FAK can be activated by thrombin, Go12, Go13, and Go4 through both Rho-dependent and Rho-independent mechanisms and that PDZ-RhoGEF and LARG can in turn be tyrosine-phosphorylated through FAK in response to thrombin, thereby enhancing the activation of Rho in vivo. These data indicate that FAK may act as a component of a positive feedback loop that results in the sustained activation of Rho by GPCRs, thus providing evidence of the existence of a novel biochemical route by which tyrosine kinases may regulate the activity of Rho through the tyrosine phosphorylation of RGL-containing RhoGEFs.

The Rho family of GTPases, including Rho, Rac, and Cdc42, comprises a large branch within the Ras superfamily of small GTP-binding proteins. Among them, Rho plays an important role in controlling the organization and dynamic remodeling of the actin-based cytoskeleton as well as in gene expression regulation. For example, when microinjected into Swiss 3T3 fibroblasts, activated Rho rapidly induces the formation of actin stress fibers and focal contacts (1), and activated alleles of Rho induce expression from the serum response element (SRE) through the activation of the serum response factor (2). Furthermore, the involvement of Rho proteins in intracellular membrane trafficking such as phagocytosis, endocytosis, and secretory vesicle transport has been also reported (3).

The functional activity of Rho, like other small G proteins, is tightly regulated in vivo by proteins that control its GDP/GTP bound state. Whereas guanine nucleotide exchange factors (GEFs) promote the exchange of GDP for GTP bound to Rho and activate its function, GTPase-activating proteins (GAPs) increase the low intrinsic rate of GTP-hydrolysis of this small GTPase, thereby negatively regulating its function (4). GEFs for Rho family G proteins, such as Dbl, Ost, Lfc, Lbc, Vav, Ect2, Tim, and Net, have been identified because of their ability to transform murine fibroblasts in standard focus-formation assays. These proteins share a common structural motif consisting of a 250-amino acid stretch of sequence similarity with Dbl, known as the DH domain, adjacent to a pleckstrin homology (PH) domain (5, 6). The DH domain is responsible for nucleotide exchange activity toward GTPases of the Rho family (7, 8). Because of this sequence similarity, the GEF families for Rho-related GTPases are often referred to as members of the Dbl superfamily (6).

It is interesting that it has been known for quite some time that G protein-coupled receptors (GPCRs), such as those for LPA, can stimulate Rho-dependent pathways (1, 9), but the nature of the heterotrimeric G proteins mediating this effect remained elusive. In this regard two lines of research suggest that the Go12 family of heterotrimeric G proteins controls Rho function and leads to actin cytoskeleton reorganization and cell growth. For example, Buhl et al. (10) demonstrated that activated Go12 and Go13, but not Go4, or different combinations of β- and γ-subunits, mimicked the effect of activated

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The abbreviations used are: SRE, serum response element; RGS, regulators of G protein signaling; RGL, RGS-like; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; DH, Dbl homology; PH, pleckstrin homology; GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; FRY2, proline-rich tyrosine kinase 2; FAK, focal adhesion kinase; GFP, green fluorescent protein; HA, hemagglutinin-β- and γ-subunits, mimicked the effect of activated go12 and go13, but not go4, or different combinations of β- and γ-subunits, mimicked the effect of activated.
Tyrosine Kinases Regulate RGL-containing RhoGEFs

RhoA on stress fibers and focal adhesion assembly; and Fromm et al. (11) provided evidence that heterotrimeric G proteins of the G_{12/13} family can promote the transcriptional activation of the serum response factor and cellular transformation through Rho. How G_{12} and G_{13} can, in turn, regulate Rho was not known. Recently available evidence indicates that a novel family of DH domain-containing proteins that includes p115RhoGEF, PDZ-RhoGEF, and LARG transduces signals from G_{12/13} proteins to Rho (12–14). These three GEFs contain an area of limited similarity to a conserved region of regulator of G protein signaling (RGS) in their NH2 termini. RGS proteins function as GAPs for G proteins and enhance the rate of GTP hydrolysis. Indeed, the RGS-like (RGL) domain of p115RhoGEF can accelerate (by 5–10-fold) the GTPase activity of G_{12/13} (15). In addition to this regulatory activity, the RGL domain of these RhoGEFs provides a structural motif by which G_{12} proteins can form molecular complexes with these RhoGEFs, thereby enhancing their activity (12, 16). Thus, this newly discovered family of RGL domain-containing RhoGEFs, p115RhoGEF, PDZ-RhoGEF, and LARG, may provide a direct functional link from G_{12}, G_{13}, and their coupled cell surface receptors to Rho (17).

However, the available information suggests that an additional mechanism may exist for regulating the ability of G_{12} proteins to stimulate the Rho signaling pathway. Indeed, several lines of evidence suggest that tyrosine kinases may regulate this pathway. For example, tyrosine kinase inhibitors have been shown to prevent G_{13}-induced neurite retraction and cell rounding in PC12 cells (18), as well as stress fiber formation and focal adhesion assembly induced by LPA and by G_{13} in murine fibroblasts (19). Even more directly, Kranebarg et al. (20) reported that Rho activation by LPA was blocked by treatment with tyrosine kinase inhibitors. Moreover, a recent study reported that the active form of G_{13} enhances the enzymatic activity of proline-rich tyrosine kinase 2 (PYK2) and that this non-receptor tyrosine kinase may be involved in the Rho-dependent activation of SRE induced by G_{12/13} (21). Based on these observations, it can be hypothesized that tyrosine kinase may play an important regulatory role in the biochemical route linking GPCR to Rho. However, the precise mechanism by which tyrosine kinases regulate Rho activation or its downstream effectors is still unknown.

Thrombin, which stimulates proteinase-activated G protein-coupled receptors (22), can provoke the rapid activation of non-receptor tyrosine kinases, including focal adhesion kinase (FAK) and Src family kinases (23, 24), and regulates Rho through G_{12/13} (25). In this study, we took advantage of these observations and a recently developed pull-down method to measure Rho activation (26) to explore the mechanism by which tyrosine kinases regulate the coupling of GPCRs to Rho. We present evidence that two RGL domain-containing RhoGEFs, PDZ-RhoGEF and LARG, can be phosphorylated in tyrosine residues by FAK in response to thrombin, and that FAK may be a component of a novel biochemical route regulating Rho activation by GPCRs.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Thrombin, carbachol, and genistein were obtained from Sigma. Poly-o-lysine was purchased from Roche Molecular Biochemicals. Tyrphostin A25 (AG 82) and tyrphostin A1 (AG 9) were purchased from Calbiochem.

**Expression Plasmides**—Expression plasmids for constitutively active forms of the G_{13}, G_{13}β, G_{13}γ, and G_{13}βγ and γ subunits of G proteins, m1/m2 muscarinic receptors, GFF, and β-galactosidase were described previously (13, 27–29). For expression of wild type and membrane-targeted forms of FAK, the cDNA was subcloned into pcDNA3 HA (Invitrogen) and pCEFL myr, a modified pcDNA3 expression plasmid encoding the NH2-terminal 21 amino acids of chicken c-Src, which includes the c-Src NH2-terminal myristoylation signal (pcDNA3 HA-FAK and pCEFL myr-FAK, respectively) (30). A DNA plasmid encoding a G_{0γ5} chimeric protein, in which 5 amino acids at the carboxyl terminus of G_{0γ} was replaced by the corresponding sequence of G_{0γ5}, was a gift from Dr. B. Conklin (31). A DNA encoding a G_{13}chimaera, in which 5 amino acids at the carboxyl terminus of G_{13} were replaced by the corresponding sequence of G_{13} (AG 9), was prepared by a chain reaction amplification using pcDNA3 HA-G_{13} as a template, and the resulting DNA was subcloned into the pCEFL HA vector as a BgIII-EcoRI fragment (32). Reporter plasmids that express the chloramphenicol acetyltransferase (CAT) gene under the control of the mutant form of the SRE from the c-fos promoter lacking the ternary complex factor binding (pSREmutL) as well as an expression vector for the C3 domain of AU1 were kindly provided by Dr. R. Treisman (2). Plasmids expressing AU1-tagged PDZ-RhoGEF, AU1-tagged LARG, and the deleted mutant form of PDZ-RhoGEF (Δ127, Δ238, Δ702, Δ956) were described previously (13, 14). cDNAs encoding amino acids 1–1160 of PDZ-RhoGEF (Δ-C) and 702–1160 of PDZ-RhoGEF (DH/PF) were generated by polymerase chain reaction amplification using pCEFL PDZ-RhoGEF AU1 as a template. Plasmid expressing AU1-tagged p115RhoGEF was subcloned into pCEFL-AU1 vector from pCAN-Myr-F1151 provided by Dr. T. Kozasa (15). pGEX expression vector encoding the glutathione S-transferase (GST) fusion protein that contains the isolated GTP-dependent binding domain of the Rhoa effector rhoetkin (rhoetkin RBD) was provided by Dr. S. Narumiya (33).

**Cell Lines and Transfection**—Human embryonic kidney 293T (HEK-293T) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Tissue culture plates were treated with phosphate-buffered saline containing 20 mg/ml poly-lysine for 15 min before seeding the cells to prevent them from detaching from the plates when in serum-free conditions. Cells were transfected using LipofectAMINE Plus™ reagent (Invitrogen) according to the manufacturer’s protocol.

**Reporter Gene Assay**—The SRE activity was determined as described previously (13, 28). Briefly, HEK-293T cells plated in a 6- or 24-well plate were transfected with the indicated plasmids together with 100 or 60 ng of pcDNA3-β-galactosidase, a plasmid expressing the enzyme β-galactosidase, and 100 or 60 ng of pSREmutL, the reporter plasmid expressing a CAT gene under the control of the mutant SRE lacking a ternary complex factor binding site, respectively. After transfection, cells were cultured for 24 h in serum-free Dulbecco’s modified Eagle’s medium and were then stimulated with the indicated ligands for an additional 6 h and lysed using reporter lysis buffer (Promega). CAT activity was assayed in the cell extracts by incubation at 37 °C for 30 min in the presence of 0.25 μCi of [14C]chloramphenicol (100 μCi/mmol, New England Nuclear) and 140–200 μg/ml butyryl-CoA (Sigma) in 0.25 M Tris-HCl, pH 7.4. Labeled butyrylated products were extracted using a mixture of xylene and 2,6,10,14-tetramethyl-pentadecane (ratio 1:2), and radioactivity was counted. β-Galactosidase activity present in each sample was assayed by a colorimetric method and was used to normalize for transfection efficiency.

**Western Blots and Immunoprecipitations**—Cells were lysed at 4 °C in a buffer containing 25 mM HEPES, pH 7.5, 0.3 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM β-glycero-phosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin, and insoluble material was removed by centrifugation. For co-immunoprecipitation, lysates were incubated for 1 h at 4 °C with the specific antibody against AU1 or FAK as indicated. Immunocomplexes were recovered with the aid of Gamma-bind Sepharose beads (Amersham Biosciences, Inc.). Lysates containing ~50 mg of total cellular protein or immunoprecipitates with the indicated antibodies were analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis and visualized by enhanced chemiluminescence detection (Amersham Biosciences Inc.) using goat anti-mouse (Cappel), goat anti-rabbit (Cappel), or donkey anti-goat (Santa Cruz Biotechnology) IgGs coupled to horseradish peroxidase as a secondary antibody. Antibodies against FAK (C20), PYK2 (N19), and RhoA (26C4) were purchased from Santa Cruz Biotechnology. Monoclonal anti-phosphotyrosine (PY99) was purchased from Upstate Biotechnology and Santa Cruz Biotechnology, respectively. Monoclonal antibody against AU1-epitope was purchased from BABCO.

**In Vivo Rho GTPase Assay—**The RhoA activity was assessed by a modified method described elsewhere (26). Briefly, HEK-293T cells were transfected with the indicated plasmids. After serum starvation for 24 h, cells were lysed at 4 °C in a buffer containing 20 mM HEPES, pH 7.4, 0.1 mM NaCl, 1% Triton X-100, 10 mM EGTA, 40 mM β-glycero-phosphate, 20 mM MgCl2, 1 mM Na2VO3, 1 mM dithiothreitol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phen-
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Activation of Rho by Thrombin: Effects of Tyrosine Kinase Inhibitors

To begin exploring the nature of pathway linking GPCRs to Rho, we first asked whether thrombin was able to stimulate Rho activity using a recently developed in vivo Rho guanine nucleotide exchange assay (26) that reveals the levels of GTP-bound form of endogenous Rho directly. For these in vivo Rho activation assays, HEK-293T cells were stimulated by thrombin and lysates were incubated with GST fusion protein including the Rho-binding domain (RBD) of rhotekin previously bound to glutathione-Sepharose beads. The levels of the GTP-bound form of Rho associated with GST-rhotekin-RBD were quantified by Western blot analysis using an anti-Rho antibody. As shown in Fig. 1A, a detailed time course analysis of Rho stimulation by thrombin in HEK-293T cells revealed the rapid and potent activation of Rho by thrombin, which was

**RESULTS**

**Activation of Rho by Thrombin: Effects of Tyrosine Kinase Inhibitors**—To begin exploring the nature of pathway linking GPCRs to Rho, we first asked whether thrombin was able to stimulate Rho activity using a recently developed in vivo Rho guanine nucleotide exchange assay (26) that reveals the levels of GTP-bound form of endogenous Rho directly. For these in vivo Rho activation assays, HEK-293T cells were stimulated by thrombin and lysates were incubated with GST fusion protein including the RBD of rhotekin previously bound to glutathione-Sepharose beads. The levels of the GTP-bound form of Rho associated with GST-rhotekin-RBD were quantified by Western blot analysis using an anti-Rho antibody. As shown in Fig. 1A, a detailed time course analysis of Rho stimulation by thrombin in HEK-293T cells revealed the rapid and potent activation of Rho by thrombin, which was

**Fig. 1. Activation of Rho by thrombin: effect of genistein.** A, HEK-293T cells were grown in 10-cm plates to 80% confluence and serum-starved overnight before being subjected to stimulation. Cultures were stimulated by thrombin (5 units/ml) for the indicated times. Lysates were incubated with GST-RBD beads for 1 h. The beads were washed, and the total amount of Rho in each cell lysate and Rho bound to the beads was analyzed by Western blotting (WB) with a monoclonal antibody against RhoA. Data are expressed as fold increase with respect to cells without stimulation, the value of which was taken as 1. AP, affinity-precipitated; TCL, total cell lysate. B and C, HEK-293T cells were treated with the indicated concentration of genistein for 20 min prior to stimulation with thrombin for 1 min (B) or 10 min (C). Lysates were processed for in vivo Rho guanine nucleotide exchange assay as described for panel A. D and E, HEK-293T cells were treated with the indicated concentrations of tyrphostin A25 (D) or tyrphostin A1 (E) for 3 h prior to stimulation. After stimulation with thrombin for 1 min (D and E, left panels) or 10 min (D and E, right panels), cells were lysed and processed for in vivo Rho guanine nucleotide exchange assay as described for panel A. Data are representative of 3–5 independent experiments.
Tyrosine Kinases Regulate RGL-containing RhoGEFs

Activation of Rho by Gα13L—effect of genistein. HEK-293T cells were transfected with plasmids for Gα13QL (A) or PDZ-RhoGEF DH/PH (B). 24 h later, cultures were treated with the indicated concentrations of genistein for 3 h, and levels of active Rho were analyzed in cellular lysates as described above. The total amount of Rho in each cell lysate was assessed with monoclonal antibody against RhoA. Data are expressed as fold increase with respect to cells transfected with empty expression vector and are representative of two additional experiments. AP, affinity-precipitated; WB, Western blot; TCL, total cell lysate.

Fig. 2. Activation of Rho by Gα13L—effect of genistein. HEK-293T cells were transfected with plasmids for Gα13QL (A) or PDZ-RhoGEF DH/PH (B). 24 h later, cultures were treated with the indicated concentrations of genistein for 3 h, and levels of active Rho were analyzed in cellular lysates as described above. The total amount of Rho in each cell lysate was assessed with monoclonal antibody against RhoA. Data are expressed as fold increase with respect to cells transfected with empty expression vector and are representative of two additional experiments. AP, affinity-precipitated; WB, Western blot; TCL, total cell lysate.

demonstrable as early as 1 min after agonist addition, and was attenuated within 10 min but remained above basal levels for several hours (not shown). To explore the correlation between Rho activation by thrombin and tyrosine phosphorylation, we studied whether this activation was impaired by pretreatment with tyrosine kinase inhibitors. For these experiments, we used both genistein and tyrphostins. Genistein is a soy-derived isoflavone known to bind to the ATP binding site of tyrosine kinases and inhibit the activity of these ATP-utilizing enzymes with high specificity (34). Tyrphostins are a series of compounds known to bind to the substrate-binding site of tyrosine kinases and have distinct inhibitory activities in various tyrosine kinase systems (35). Initially, we found that genistein had no effect on activation of Rho after 1 min stimulation by thrombin (Fig. 1B) but clearly diminished the levels of active Rho after 10 min of thrombin stimulation (Fig. 1C). Next, we tested the effect of tyrphostin A25, a broad tyrosine kinase inhibitor that has been reported to block Gα13Q-induced neurite retraction in PC12 cells (18) and stress fiber formation stimulated by LPA in Swiss 3T3 cells (36). We found that tyrphostin A25 had no effect on activation of Rho after 1 min stimulation by thrombin but clearly diminished the activated Rho after 10 min of thrombin stimulation (Fig. 1D). In contrast, tyrphostin A1, an inactive, related compound that can serve as a negative control for tyrphostins, had no effect on the activation of Rho by thrombin at both early and late time points (Fig. 1E). These data suggested that tyrosine kinases contribute to Rho activation by thrombin not during the early phase immediately after thrombin addition but during the more sustained and prolonged delayed phase.

Activation of Rho by Gα13L Is Reduced by Tyrosine Kinase Inhibitors—As the thrombin receptor PAR-1 (proteinase-activated G protein-coupled receptor-1) is reported to couple to the Gαs, Gα12/13, and Gq families of heterotrimeric G protein α subunits (25, 37, 38) and Gα12/13 are known to be functionally linked to Rho (11), we next investigated the contribution of tyrosine phosphorylation to the ability of Gα12/13 to stimulate Rho. Indeed, expression of activated Gα12 and Gα13 potently elevated the steady state level of endogenous GTP-bound Rho (Fig. 2A and data not shown), and treatment with genistein diminished the Rho activity induced by the activated form of Gα13 in a dose-dependent manner (Fig. 2A). In contrast, we did not detect any effect of genistein on Rho activation when induced by the DH/PH domain of PDZ-RhoGEF, which served as a control (Fig. 2B). Together, these data suggested a regulatory role for tyrosine kinases in the pathway linking GPCRs and Gα12/13 to Rho.

Thrombin Activates FAK in HEK-293T Cells—Because these data suggested that tyrosine kinases are involved in the activation of Rho by thrombin in HEK-293T cells, we next explored which tyrosine kinase was involved in this pathway. As the tyrosine kinase PYK2 was reported to participate in SRE activation by Gα13 in HeLa cells (21), we viewed PYK2 and its closely related tyrosine kinase, FAK, as good candidates to mediate this response. Initially, we examined the expression of FAK and PYK2 in HEK-293T cells. FAK was endogenously expressed in HEK-293T cells, but PYK2 was not detectable in these cells, although transfected PYK2 was readily demonstrable when used as a control (Fig. 3A). Based on these results, we asked whether thrombin could stimulate FAK activity in these cells. For these experiments, endogenous FAK was immunoprecipitated with anti-FAK antibody, and phosphorylated FAK was detected by Western blotting using anti-phosphotyrosine antibodies. When stimulated with thrombin, tyrosine phosphorylation of FAK was evident as early as 5 min after addition of thrombin and peaked at ∼10 min after stimulation (Fig. 3B). These data suggest that in HEK-293T cells endogenous FAK is activated in response to thrombin and that FAK may represent a good candidate for regulation of the molecular pathways initiated by thrombin receptors.

FAK Activation by Gα13L and Gα12/13-coupled Receptors—We next investigated which G proteins can stimulate FAK. As an approach, we first used the expression of GTPase-deficient mutationally activated forms of G protein α subunits, which can activate effector pathway by obviating the need for receptor stimulation (39). We transfected HEK-293T cells with GTPase-
deficient mutants for Ga\textsubscript{12}, Ga\textsubscript{13} as well as Ga\textsubscript{12}, Ga\textsubscript{13}, and β\textsubscript{y} subunits (Fig. 4A). As shown in Fig. 4A, the activated mutants of Ga\textsubscript{12} and Ga\textsubscript{13} stimulated FAK tyrosine phosphorylation efficiently. The activated mutant of Ga\textsubscript{12} slightly activated FAK, but Ga\textsubscript{a} and Gβ\textsubscript{y}, had no demonstrable effect under our experimental conditions. To investigate further the coupling specificity of GPCRs linked to FAK activation, we chose to use a reconstituted system consisting of the expression of m1 and m2 muscarinic receptors, which transmit signals through Ga\textsubscript{q} and Ga\textsubscript{i}, heterotrimeric G proteins, respectively. As a control, both receptors stimulated mitogen-activated protein kinase effectively (Ref. 40 and data not shown), but only m1 receptors lead to FAK phosphorylation as previously reported (41). Thus, coupling to Ga\textsubscript{i} does not appear to be sufficient to stimulate FAK. These results enabled us to take advantage of the finding that a Ga\textsubscript{q}, Ga\textsubscript{13} chimera, where a carboxy-terminal region of Ga\textsubscript{q} or Ga\textsubscript{13} is replaced by the corresponding region of Ga\textsubscript{i}, can be stimulated by Ga\textsubscript{i}-coupled receptors and is able to transmit Ga\textsubscript{i}-and Ga\textsubscript{13}-mediated signaling pathways, respectively (31, 32). Thus, upon co-expression of these Ga\textsubscript{q}, Ga\textsubscript{13} chimeras together with a Ga\textsubscript{i}-coupled receptor, m2, on and off Ga\textsubscript{i} or Ga\textsubscript{13}-mediated signaling can now be controlled by agonist such as carbachol addition. As shown in Fig. 4B, in cells expressing either the Ga\textsubscript{q}, Ga\textsubscript{13} chimera, m2 receptor stimulation resulted in FAK phosphorylation. These data indicate that both Ga\textsubscript{i} and Ga\textsubscript{12/13} classes of G proteins are able to signal to FAK and might participate in FAK activation by GPCRs, such as those receptors activated by thrombin.

Stimulation of FAK by Both Thrombin and Ga\textsubscript{12/13} Subunits Involves Rho-dependent and -independent Mechanisms—Thrombin stimulation, as well as activated forms of Ga\textsubscript{12/13} and Ga\textsubscript{i} subunits, induces activation of FAK and Rho. These observations raised the possibility that Rho and FAK could be part of a signaling pathway involving FAK downstream from Rho, as previously suggested (42). To explore this possibility, we made use of a DNA construct that expresses the botulinum C3 exoenzyme, which ADP-ribosylates RhoA at asparagine 41, thereby preventing the exchange of GDP by GTP and retaining RhoA in its GDP-bound inactive form (2, 43). As a control, we measured the activation of a reporter plasmid containing a mutated SRE, which eliminates the ternary complex factor-binding site and is potently activate by Rho (2). As seen in Fig. 5A, cotransfection with a plasmid expressing the C3 toxin could only partially reduce FAK activation by thrombin (Fig. 5A, upper panel). However, even low amounts of C3 toxin were sufficient to abolish the SRE response to thrombin. Similarly, C3 abolished the ability of activated Ga\textsubscript{q}, Ga\textsubscript{12}, and Ga\textsubscript{13} to

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**Fig. 3.** *Thrombin activates FAK in a time-dependent manner.* A, HEK-293T cells were transfected with expression plasmids for GFP as a control (C), myr-FAK, or PYK2. Lysates were prepared as described under “Experimental Procedures.” The expression level of FAK and PYK2 in extracts from transfected cells was determined by Western blotting using anti-FAK (left panel) or anti-PYK2 (right panel) antibodies, respectively. TCL, total cell lysate; WB, Western blot. B, HEK-293T cells were grown in 10-cm plates to 80% confluence and serum-starved overnight before being subjected to stimulation with thrombin (5 units/ml) for the indicated times. Subsequently cells were quickly lysed on ice, and cell lysates were immunoprecipitated with rabbit anti-FAK serum (IP). Activated FAK was detected by Western blot analysis with an anti-phosphotyrosine antibody. The amount of FAK in each cell lysate was assessed by Western blot analysis with an anti-FAK antibody.

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**Fig. 4.** *Effect of activated mutants of Ga subunits of heterotrimeric G proteins and Gβγ dimers on the activation of FAK.* A, HEK 293T cells were transfected with plasmids expressing β-galactosidase (C), Ga\textsubscript{12}, Ga\textsubscript{13}, Ga\textsubscript{12}, Ga\textsubscript{13}, Ga\textsubscript{12}, Ga\textsubscript{13}, Ga\textsubscript{12}, Ga\textsubscript{13}, or β\textsubscript{y} subunits as indicated. 24 h later, lysates were immunoprecipitated with anti-β-galactosidase (C) antibody and used for Western blot (WB) analysis. Phosphorylated FAK was determined by Western blotting using anti-FAK antibody (Ref. 40 and data not shown), but only m1 receptors lead to FAK phosphorylation as previously reported (41). Thus, coupling to Ga\textsubscript{i} does not appear to be sufficient to stimulate FAK. These results enabled us to take advantage of the finding that a Ga\textsubscript{q}, Ga\textsubscript{13} chimera, where a carboxy-terminal region of Ga\textsubscript{q} or Ga\textsubscript{13} is replaced by the corresponding region of Ga\textsubscript{i}, can be stimulated by Ga\textsubscript{i}-coupled receptors and is able to transmit Ga\textsubscript{i}-and Ga\textsubscript{13}-mediated signaling pathways, respectively (31, 32). Thus, upon co-expression of these Ga\textsubscript{q}, Ga\textsubscript{13} chimeras together with a Ga\textsubscript{i}-coupled receptor, m2, on and off Ga\textsubscript{i} or Ga\textsubscript{13}-mediated signaling can now be controlled by agonist such as carbachol addition. As shown in Fig. 4B, in cells expressing either the Ga\textsubscript{q}, Ga\textsubscript{13} chimera, m2 receptor stimulation resulted in FAK phosphorylation. These data indicate that both Ga\textsubscript{i} and Ga\textsubscript{12/13} classes of G proteins are able to signal to FAK and might participate in FAK activation by GPCRs, such as those receptors activated by thrombin.
These data are consistent with the ability of Gα12 and Gα13 to induce Rho and with the recent observation that Gα12 can stimulate Rho potently in this cell type. Of interest, in parallel experiments C3 toxin only partially reduced FAK tyrosine phosphorylation (Fig. 5B). Taken together, these observations suggest that thrombin, and more specifically Gαq, Gα12, and Gα13, can use both Rho-dependent and -independent mechanisms to activate FAK.

**Activation of Rho by FAK**—Based on the prior results, we asked whether FAK could act upstream rather than downstream from Rho. Using an activated form of FAK, we first observed that FAK could activate expression from the SRE reporter plasmid and that this activation was highly sensitive to the inhibitory effect of C3 toxin (Fig. 6A). Next, we studied whether FAK may have a direct effect on Rho activation, using the in vivo Rho guanine nucleotide exchange assay. As shown in Fig. 6B, expression of the activated form FAK effectively increased the amount of the GTP-bound form of Rho by 3–4-fold without causing any changes in the expression levels of endogenous Rho. Activation of Rho by FAK was prevented by tyrosine kinase inhibitors (data not shown). Collectively, these data indicate that FAK can effectively activate Rho and Rho-dependent pathways.

**Tyrosine Phosphorylation of RGL Domain-containing RhoGEFs by FAK**—To begin addressing the mechanism by which FAK activate Rho, we next explored the possibility that FAK causes the tyrosine phosphorylation of RGL domain-containing RhoGEFs (17) including PDZ-RhoGEF, LARG, and p115RhoGEF. As an approach, we co-transfected HEK-293T cells with epitope-tagged forms of these RhoGEFs and the activated form of FAK. After immunoprecipitation with anti-epitope antibodies, we examined the level of tyrosine-phosphorylated RhoGEFs using anti-phosphotyrosine antibodies (Fig. 7). We found that the activated form of FAK effectively phosphorylates two RGL domain-containing RhoGEFs, PDZ-RhoGEF and LARG, but not p115RhoGEF. As PDZ-RhoGEF is highly related to LARG (14), we chose to focus on the former to investigate further the functional consequences of tyrosine phosphorylation of these RhoGEFs by FAK.

**Tyrosine Phosphorylation of PDZ-RhoGEF Enhances Rho Activation**—We next performed in vivo Rho guanine nucleotide exchange assays to determine whether FAK can affect the activity of PDZ-RhoGEF on Rho. HEK-293T cells were transfected with PDZ-RhoGEF together with a plasmid control or the activated form of FAK. As shown in Fig. 8, Rho was activated by PDZ-RhoGEF overexpression, and this Rho activity was increased further in cells co-transfected with the activated form of FAK without affecting the level of expression of PDZ-RhoGEF (data not shown). Thus, these results indicate that activated FAK promotes the tyrosine phosphorylation of PDZ-RhoGEF and enhances Rho activation in vivo.

**Thrombin Induced PDZ-RhoGEF Phosphorylation in a Time-dependent Manner**—As thrombin stimulates FAK and an activated form of FAK phosphorylates PDZ-RhoGEF, we next explored whether thrombin can induce tyrosine phosphorylation
of PDZ-RhoGEF. As shown in Fig. 9A, thrombin stimulation could effectively induce PDZ-RhoGEF tyrosine phosphorylation in a time-dependent manner. PDZ-RhoGEF tyrosine phosphorylation in response to thrombin was even more prominent after co-transfecting the cells with the wild type form of FAK, which by itself does not increase the basal level of phosphorylated PDZ-RhoGEF but facilitates the detection of phosphorylated forms of this RhoGEF. Under these conditions, the earliest time point at which we observed this phosphorylation was around 3–5 min (Fig. 9B and data not shown), which was consistent with the time course of FAK activation by thrombin but delayed with respect to the early peak of Rho activation (see above).

**Delineation of the Phosphorylation-responsive Region**—To elucidate which structural feature of PDZ-RhoGEF is the target for tyrosine phosphorylation, we made use of various truncated mutants of PDZ-RhoGEF, examining them for FAK-dependent tyrosine phosphorylation in response to thrombin by using experimental conditions similar to those described above (Fig. 10). When HEK-293T cells were stimulated with thrombin in the presence of wild type FAK, NH2-terminal deletions of PDZ-RhoGEF (∆1–127, ∆1–238, ∆1–702, and ∆1–956) retained their tyrosine phosphorylation. However, ∆C, a deletion form lacking the carboxyl terminus of PDZ-RhoGEF, nearly lost its tyrosine phosphorylation (Fig. 10B). These data suggest that the carboxyl-terminal domain plays an essential role for PDZ-RhoGEF phosphorylation by FAK such as providing the tyrosine phosphoacceptor sites or structural features required for substrate recognition. These possibilities are under current investigation.

**FAK Blockade Diminishes PDZ-RhoGEF Tyrosine Phosphorylation**—To confirm the correlation between PDZ-RhoGEF tyrosine phosphorylation and the FAK-FAK interaction, we examined the effect of FAK inhibition on PDZ-RhoGEF tyrosine phosphorylation. HEK-293T cells were transfected with plasmids for PDZ-RhoGEF-AU1, LARG-AU1, or p115RhoGEF-AU1 together with expression plasmids carrying cDNAs for GFP or myr-FAK as indicated. Lysates were prepared as described under “Experimental Procedures.” Lysates were immunoprecipitated (IP) with anti-AU1 antibody and subjected to Western blot (WB) analysis with anti-phosphotyrosine antibody. The expression level of PDZ-RhoGEF-AU1, LARG-AU1, or p115RhoGEF-AU1 in total cell lysates was determined by Western blotting using an anti-AU1 antibody.

![Graph showing activation of Rho by FAK](image)

**FIG. 6. Activation of Rho by FAK.** A, HEK-293T cells plated in 24-well plates were cotransfected with pSREmutL and pCMV-β-galactosidase plasmid DNAs as well as with expression vectors carrying cDNAs for GFP as a control and myr-FAK with or without expression plasmids for C3 toxin, as indicated. The next day, the cells were collected, and the lysates were assayed for CAT activity. The data represent CAT activity normalized by the β-galactosidase activity present in each cellular lysate expressed as fold induction with respect to control cells and are the means ± S.E. of triplicate samples from a typical experiment. B, HEK-293T cells were transfected with plasmids for myr-FAK, β-galactosidase as a control (C), or GsQL. 24 h later, lysates were incubated with GST-RBD beads for 1 h. The beads were washed, and the bound protein was analyzed by Western blotting (WB) with a monoclonal antibody against Rhoa. The total amount of Rhoa in each cell lysate was assessed with monoclonal antibody against Rhoa. Data are expressed as fold increase with respect to cells transfected with plasmids for β-galactosidase, for which the value was taken as 1. Data are representative of three independent experiments. AP, affinity-precipitated.

![Graph showing phosphorylation of PDZ-RhoGEF and LARG by FAK](image)

**FIG. 7. Phosphorylation of PDZ-RhoGEF and LARG by FAK.** HEK-293T cells were transfected with expression vectors for PDZ-RhoGEF-AU1, LARG-AU1, or p115RhoGEF-AU1 together with expression plasmids carrying cDNAs for GFP or myr-FAK as indicated. Lysates were prepared as described under “Experimental Procedures.” Lysates were immunoprecipitated (IP) with anti-AU1 antibody and subjected to Western blot (WB) analysis with anti-phosphotyrosine antibody. The expression level of PDZ-RhoGEF-AU1, LARG-AU1, or p115RhoGEF-AU1 in total cell lysates was determined by Western blotting using an anti-AU1 antibody.
rosine phosphorylation and its activation by FAK, we studied whether these phenomena are impaired by direct blockade of FAK. As an approach, we used the expression of the FAK-related non-tyrosine kinase (FRNK), which is a noncatalytic carboxyl-terminal domain of FAK and is known to prevent the localization of FAK to focal adhesion sites and to reduce FAK tyrosine phosphorylation (44). First, as a control, we studied the effect of FRNK on PDZ-RhoGEF phosphorylation by active FAK. We found that FRNK reduces PDZ-RhoGEF phosphorylation in a dose-dependent manner (Fig. 11A), confirming the effectiveness of this approach. In line with this observation, FRNK also reduced PDZ-RhoGEF phosphorylation by thrombin stimulation (data not shown). Next, we explored the effect FRNK on Rho activation by thrombin. We found that FRNK had no effect on the early phase of activation of Rho after 1 min of stimulation with thrombin (Fig. 11B) but dimin-

**DISCUSSION**

In the present study, we present evidence of the existence of a novel biochemical route regulating the signaling pathway from GPCRs to Rho. We found that tyrosine kinases contribute to Rho activation by thrombin, not during the early phase immediately after thrombin addition but during the more sustained and prolonged delayed phase. Focusing on FAK, a non-receptor tyrosine kinase endogenously expressed in HEK-293T cells, we observed that its activity can be regulated by thrombin receptors and that, in turn, FAK contributes to the prolonged activation of Rho by these G protein-linked receptors through the tyrosine phosphorylation of two members of the RGL domain-containing RhoGEFs, PDZ-RhoGEF and LARG.
Available evidence indicated that thrombin can induce the activation of FAK through Go12/13 and Go5, and that this pathway involves Rho-dependent and Rho independent mechanisms. In this regard, we observed in preliminary experiments that activated forms of Rac and Cdc42 were also effective in stimulating FAK tyrosine phosphorylation. Thus, we can envision that although FAK can act downstream of Rho (42), thrombin receptors and heterotrimeric G proteins may activate FAK through both Rho-dependent and -independent mechanisms, the latter likely occurring through additional GTPases of the Rho family. On the other hand, early reports describing tyrosine kinase inhibitors as blocking Rho activation by LPA and Go12/13 (18–20) suggested that tyrosine kinases can participate in the regulation of Rho by GPCRs. Moreover, it was reported that adhesion of fibroblasts to fibronectin, which stimulates FAK, potently promotes prolonged Rho activation (26). These data suggest that FAK can also act upstream of Rho. Indeed, our present results indicate that active forms of FAK can promote the accumulation of Rho in the GTP-bound, active state. These observations raise the possibility of the existence of a positive feedback regulation between Rho and FAK; that is, Rho stimulates FAK, and once activated, FAK enhances the levels of active Rho (Fig. 12). This possibility is consistent with the observation that Rho activation is earlier than that of FAK and with evidence obtained by the use of tyrosine kinase inhibitors and FAK-inhibiting molecules that supports the notion that FAK does not contribute to the early phase of Rho activation but probably participates by enhancing the more sustained and prolonged delayed phase.

We note with interest that available data suggest tyrosine kinases may have two opposing effects on Rho function. For example, FAK may also suppress Rho function upon focal contact formation (26, 45). In this regard, it is intriguing that two Rho-GAPs interact with focal adhesion components or with FAK. For example, p190RhoGAP, which has been shown to inhibit Rho-induced stress fiber formation (46, 47) can be recruited to focal contacts (48) and is phosphorylated by tyrosine kinases, which negatively regulate Rho function (49). GRAF (GAP for Rho associated with FAK) is reported to interact directly with FAK using its SH3 (Src homology 3) domain (50). These Rho-GAPs may interact with FAK directly or indirectly, and they play an inhibitory role for Rho activation. Thus, it is possible that after a rapid activation of Rho through the direct interaction of heterotrimeric G proteins with RhoGEFs, FAK
may play a biphasic role for Rho, that is, on the one hand enhancing Rho activity through the tyrosine phosphorylation of RhoGEFs and on the other hand impairing Rho activity through the recruitment of RhoGAPs. These seemingly opposing functions for FAK may facilitate the fine-tuning of the pathways controlling Rho activation. As many of the intervening molecules exhibit a distinct subcellular distribution, it is also possible that the complex interplay of GEFs and GAPs and their regulation by FAK may participate in the appropriate localization of Rho function to specific subcellular regions.

How tyrosine phosphorylation of PDZ-RhoGEF and LARG contributes to their activation is at present unknown. Indeed, efforts are currently under way to dissect out the contribution of G proteins and tyrosine kinases in the activation of the GEF activity of PDZ-RhoGEF toward Rho in vivo reconstitution systems. Thus far, the best example of a GEF in which activity is tightly regulated by tyrosine phosphorylation is Vav-1, which we found in previous studies to require tyrosine phosphorylation for its activity as a RacGEF in vitro (51) and in vivo (52). Vav-2, a protein structurally related to Vav-1, was also reported to exhibit GEF activity in response to tyrosine phosphorylation (53). In the case of Vav-1, phosphorylation of Tyr-174, which is an integral part of an auto inhibitory interface, by Syk or Src-like kinases causes the NH2-terminal peptide to become unstructured and released from the DH domain, resulting in its activation (54). More recently, proto-Dbl was shown to exhibit GEF activity in response to tyrosine phosphorylation (55). In the case of proto-Dbl, it is reported that an NH2-terminal peptide becomes unstructured and released from the DH domain, resulting in its activation (55).

For PDZ-RhoGEF and LARG, this regulation may be even more complex, as they exhibit an RGS like domain that provides a structural feature by which they can interact with G proteins and may strengthen the Rho signal provoked by GPCRs, thus providing evidence that phosphorylation may play a biphasic role for Rho, that is, on the one hand enhancing Rho activity through the tyrosine phosphorylation of RhoGEFs and on the other hand impairing Rho activity through the recruitment of RhoGAPs. These seemingly opposing functions for FAK may facilitate the fine-tuning of the pathways controlling Rho activation. As many of the intervening molecules exhibit a distinct subcellular distribution, it is also possible that the complex interplay of GEFs and GAPs and their regulation by FAK may participate in the appropriate localization of Rho function to specific subcellular regions. The observations also raise the possibility that these RhoGEFs may play a broader role in signal transduction, as they could provide a link to Rho to certain cell surface receptors that either exhibit tyrosine kinase activity or stimulate cytoplasmic tyrosine kinases, in addition to their better understood role in signaling by G protein-linked receptors.
Tyrosine Kinases Regulate RGL-containing RhoGEFs

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