Identification of Potential Mechanisms for Regulation of p115 RhoGEF through Analysis of Endogenous and Mutant Forms of the Exchange Factor*

Rho GTPases play a fundamental role in numerous cellular processes that are initiated by extracellular stimuli including agonists that work through G protein-coupled receptors. A direct pathway for such regulation was elucidated by the identification of p115 RhoGEF, an exchange factor for RhoA that is activated through its RGS domain by Ga13. Endogenous p115 RhoGEF was found mainly in the cytosol of serum-starved cells but partially localized to membranes in cells stimulated with lysophosphatidic acid. Overexpressed p115 RhoGEF was equally distributed between membranes and cytosol; either the RGS or pleckstrin homology domain was sufficient for this partial targeting to membranes. Removal of the pleckstrin homology domain dramatically reduced the in vitro rate of p115 RhoGEF exchange activity. Deletion of amino acids 252–288 in the linker region between the RGS domain and the Dbl homology domain or of the last 150 C-terminal amino acids resulted in non-additive reduction of in vitro exchange activity. In contrast, p115 RhoGEF pieces lacking this extended C terminus were over 5-fold more active than the full-length exchange factor in vivo. These results suggest that p115 RhoGEF is inhibited in the cellular milieu through modification or interaction of inhibitory factors with its C terminus. Endogenous p115 RhoGEF that was immunoprecipitated from cells stimulated with lysophosphatidic acid or sphingosine 1-phosphate was more active than when the enzyme was immunoprecipitated from untreated cells. This indicates an additional and potentially novel long-lived mechanism for regulation of p115 RhoGEF by G protein-coupled receptors.

Cell movement and shape change are necessary in complex biological processes such as morphogenesis, cell division, wound healing, cell adhesion, and phagocytosis. Such cellular behavior is directed by precise monitoring of the extracellular milieu through contacts with extracellular matrix and concentrations of circulating stimuli. The actin cytoskeleton plays a dynamic role in the coordination of these signals as evidenced by its role in clustering and dispersal of focal adhesions, events that are regulated through the monomeric Rho GTPases by heterotrmeric G protein-coupled receptors, receptor tyrosine kinases, and integrin receptors (1–6).

Rho GTPases compose a family of monomeric G proteins in the superfamily of Ras GTPases; other families include the Arf, Rab, Ran, Ras, and Sar GTPases. The mammalian Rho-like family of GTPases, which range in molecular mass from 21 to 28 kDa, is further divided into at least three subfamilies, Rho (RhoA–E), Rac (Rac1–3 and RhoG), and CDC42. Although primarily associated with regulation of cytoskeletal rearrangements, Rho GTPases also convey signals controlling gene transcription, cell cycle progression, and cytokinesis (7–9).

All monomeric G proteins cycle between an active GTP-bound state and an inactive GDP-bound form. Dissociation of GDP is potentiated by guanine nucleotide exchange factors (GEFs) that preferentially bind and stabilize the nucleotide-free enzyme. Association of GTP, which is in excess over GDP in the cell, promotes release from exchange factor and interaction of the activated G protein with downstream effectors. GTPase-activating proteins increase the intrinsic rate of hydrolysis of GTP bound to the G proteins and thus decrease the life span of bound GTP and the transmission time for each quantum of signaling (10).

Numerous GEFs are capable of stimulating nucleotide exchange on various members of the Rho family. Over half of these GEFs were identified in screens for proteins that could induce formation of transformed foci when expressed in mouse NIH3T3 cells. All of these proteins were found to share a conserved sequence of about 180 amino acids with the CDC42 exchange factor, Dbl, and this region is now called the Dbl homology (DH) domain (11). The importance of the DH domain in binding Rho and stimulation of nucleotide exchange is well documented (11, 12). The coordinate inability of GEFs with mutated DH domains to transform NIH3T3 cells strongly indicates that the exchange activity of this region is responsible for the cellular phenotype (12). All Rho family exchange factors also contain a pleckstrin homology (PH) domain that is C-terminal to the DH domain (11).

The heterotrimeric G12 and G13 proteins mediate signaling between the extracellular agonist LPA and RhoA-dependent signaling (6). Intracellular RhoA was found to be activated by the GTPase-deficient mutants Ga12Q (Q229L) or Ga13Q (Q226L).

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1 The abbreviations used are: Rho, Ras homology; DH, Dbl homology; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; GST, glutathione S-transferase; GEF, guanine nucleotide exchange factor; HA, hemagglutinin; HER2/3, human embryonic kidney 293; LPA, lysophosphatidic acid; PH, pleckstrin homology; S1P, sphingosine 1-phosphate; aa, amino acid; GTPyS, guanosine 5’-3-O-(thio)triphosphate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; SRE, serum response element.
but not by Gαq (Q204L) (13). Studies have also found that introduction into cells of the GTPase-deficient mutants of either Gαq13 or Gαq12 mutants causes phenotypes that are similar to those induced by treatment of cells with LPA. LPA-induced rounding of NE1–115 cells was also caused by overexpression of Gαq12 (Q229L) or Gαq13 (Q226L), and this rounding was blocked by expression of dominant negative N19-RhoA (13). Additionally, expression of active RhoA (Q63L), Gαq12 (Q229L), or Gαq13 (Q226L), but not Gαq (Q209L) or Gαq (Q204L), promoted formation of stress fibers in Swiss 3T3 cells by a process that was inhibited by the Clostridium botulinum C toxin (14). Attenuation of LPA-mediated formation of stress fibers in Swiss 3T3 cells by microinjection of antibodies against Gαq13, but not by antibodies against Gqα11 or Gαq12, provided direct evidence for the requirement of Gαq13 in this process (15).

A Rho-selective exchange factor, p115 RhoGEF, directly links Gα13 to regulation of RhoA. This exchange factor contains an N-terminal domain that is homologous to the family of RGS (regulator of G protein signaling) proteins and acts as a GTPase-activating protein for the Gαq13 and Gαq12 proteins (16). Furthermore, the activated form of Gαq13, in vitro, stimulates the ability of p115 RhoGEF to catalyze dissociation of GDP from RhoA by 3–4-fold (17). Overexpression of either Gαq13 or p115 RhoGEF has been shown to potentiate transcription of a reporter gene that is regulated by the modified serum response element (SRE.L) (18); this response element drives transcription of the molecule that are important for nucleotide exchange of the molecule and point toward a role for the C terminus in maintaining low basal activity of p115 RhoGEF within the cell.

MATERIALS AND METHODS

Expression Plasmids—The vector expressing C. botulinum C3 toxin (20) and the pRLE-TK vector for transcription of Renilla reniformis luciferase under the control of the thymidine kinase promoter (21) were provided by Melanie Cobb. The SRE.L promoter (22) was provided by Silvio Gutkind. The SRE.L promoter was inserted in front of the firefly (Photinus pyralis) luciferase reporter gene in the pGL3 basic mammalian expression vector (Promega). The cDNA encoding full-length p115 RhoGEF (23) was used for amplification of the p115 RhoGEF fragments by the polymerase chain reaction. All fragments were amplified with an N-terminal EcoRI site and a C-terminal HindIII site for cloning into the pCMV5 (provided by David Russell), pCMV5-myc (Melanie Cobb), and pGEX-KG (24) expression vectors. Fragments were transfected via the E coli RXba site from pCMV5 into pVL1392-EE, pVL1392 (Invitrogen) which has been modified to provide an N-terminal EE tag (EYPMPE) (23). The polymerase chain reaction fragment of p115 RhoGEF encoding amino acids 1–252 was cloned into pGEX-KG and pTrc D. The latter was constructed from pTrc C (Invitrogen) by replacing the intervening sequence between the His tag and the EcoRI restriction site with the intervening amino acids, MGA. The different pieces of p115 RhoGEF are named by sequence between the His tag and the sequence. The polymerase chain reaction fragment of p115 RhoGEF encoding amino acids 288–637 was expressed as a fusion protein with GST (GST-RBD) in 25 mM NaHEPES, pH 7.5, 1 mM DTT, 50 mM NaCl, and protease inhibitors. After removing cell debris by centrifugation at 100,000 × g, the resulting supernatant was passed over a glutathione-Sepharose column, and bound RhoA was eluted with the same solution containing 1% cholate. Preliny RhoA was concentrated, and cholate was removed by diluting samples in 25 mM NaHEPES, pH 7.5, 1 mM DTT, 50 mM NaCl, and protease inhibitors, followed by concentration by pressure filtration through an Amicon PM10 membrane.

Antisera—The three purified polypeptide fragments of p115 RhoGEF, aa 1–252, EE-tagged aa 288–637, and EE-tagged aa 760–912 were immediately collected by filtration through BA-85 filters (International Technological, Inc.) and 10% fetal bovine serum (FBS) (Life Technologies, Inc.) and 10% fetal bovine serum (FBS) (Life Technologies, Inc.). After centrifugation at 100,000 × g, the resulting supernatant was passed over a glutathione-Sepharose column, and bound RhoA was eluted with the same solution containing 1% cholate. Preliny RhoA was concentrated, and cholate was removed by diluting samples in 25 mM NaHEPES, pH 7.5, 1 mM DTT, 50 mM NaCl, and protease inhibitors, followed by concentration by pressure filtration through an Amicon PM10 membrane.

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Assessment of GTP-β-S Binding to RhoA—Binding of GTP-β-S to 2 μM RhoA was assayed at 30 °C in 20 μl of a solution containing 20 μM GTP-β-S, [35S]GTP-β-S (200,000 cpm), 50 mM NaHEPES, pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, and protease inhibitors. After incubation with mixed buffer at 17 °C on a rocking platform, the Sepharose beads were pelleted in a microcentrifuge for 20 s. The supernatant, which contains the unbound fraction of p115 RhoGEF, was removed, and the beads were washed three times with 400 μl of incubation buffer. p115 RhoGEF that was bound to the Sepharose beads was released by boiling in SDS-PAGE disruption buffer. The relative amounts of p115 RhoGEF bound to Sepharose beads in the free fraction were compared by immunoblot analysis using the U2762 antibody.

Subcellular Localization of Endogenous p115 RhoGEF—Endogenous p115 RhoGEF was detected in cytosol and membranes of NIH3T3 and COS cells by immunoblot analysis. Cells were grown to 90% confluence in 10-cm plates in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc.) and 10% fetal bovine serum (FBS) (Life Technologies, Inc.) and 10% fetal bovine serum (FBS) (Life Technologies, Inc.). After washing in phosphate-buffered saline, cells were collected by scraping and lysed with a glass Dounce homogenizer. Particulate debris was removed by centrifugation at 700 × g. Membranes and cytosol were then separated by centrifugation of samples at 100,000 × g. Samples of each fraction were solubilized by boiling in sample buffer, and SDS-PAGE was utilized to separate 50 μg of either membrane-
associated or cytosolic proteins. The relative amounts of p115 RhoGEF were then visualized by immunoblot analysis using the U2764 antibody.

Subcellular Localization of Recombinant p115 RhoGEF—pCMV5-myc vectors encoding fragments of p115 RhoGEF were transfected into COS cells and serum-starved in 35-mm wells. Transfections were accomplished using the Fugene method (Hoffmann-La Roche). After 24 h of incubation at 37 °C in 5% CO2, membrane and cytosolic fractions were prepared as described for endogenous p115 RhoGEF. Two micrograms of total protein derived from either membrane or cytosolic fractions were separated by SDS-PAGE, and the relative amounts of the recombinant p115 RhoGEF pieces were visualized by immunoblot analysis using an antibody directed against the Myc tag.

Serum Response Element—A mutated serum-response element (SRE), SRE-L, which lacks the C-Fos ternary complex-binding site and responds to RhoA more specifically than the wild type SRE promoter (19, 22, 28), was used to monitor activation of RhoA. Equal amounts of expression plasmid DNA, 0.5% sodium cholate, 10 mM MgCl2, 500 mM NaCl, and 0.5% LPS were added to each well. Cells were lysed and probed with the anti-RhoA monoclonal antibody. The beads were washed three times with 500 μl of wash buffer (20 mM NaHEPES, pH 7.5, 1% octyl glucoside, 0.5% sodium cholate, 10 mM MgCl2, 500 mM NaCl, and 1% Triton X-100) and protease inhibitors and bound proteins were eluted by boiling in SDS sample buffer. Proteins within the samples were separated by SDS-PAGE and examined by immunoblot analysis for the content of RhoA.

To assess differential activation of RhoA by p115 RhoGEF pieces in vitro, HEK293 cells were co-transfected using the calcium phosphate method (29) with p115 RhoGEF and HA-tagged RhoA. After 16 h of incubation at 37 °C in 5% CO2, cells were lysed and probed with the GST-RBD domain as described above. Five micrograms of GST-RBD, which was immobilized to glutathione-Sepharose beads, was added to each well. The beads were washed three times with 500 μl of cold wash buffer (20 mM NaHEPES, pH 7.5, 1% octyl glucoside, 10 mM MgCl2, 150 mM NaCl, and 1% Triton X-100) and protease inhibitors and bound proteins were eluted by boiling in SDS sample buffer. Proteins within the samples were separated by SDS-PAGE and examined by immunoblot analysis for the content of RhoA.

RESULTS

Expressed Proteins and Antisera—Initial characterization of p115 RhoGEF in vitro has established two functional motifs. The N-terminal third of the protein, which contains the RGS homology region, acts as a GTPase-activating protein for Ga12 and Ga13 (16). The rest of the protein, which contains the predicted DH and PH domains, is an effective exchange factor for the Rho GTPase (23). However, the mechanism by which Ga13 effects stimulation of exchange activity for Rho or the regulatory properties of this protein in the cellular milieu are undefined. In order to gain insight into these mechanisms, a more specific selection analysis of the protein was undertaken. The proteins were expressed either without a tag or with an N-terminal Myc tag. All three antisera could detect recombinant and endogenous p115 RhoGEF by immunoblot analysis (Figs. 2A and 5A). The U2762 (DH) and U2764(C-terminal) antibodies could also be used to detect endogenous protein by immunofluorescence (data not shown) and specifically immunoprecipitated endogenous exchange factor (Fig. 7D).

Binding of p115 RhoGEF Pieces to RhoA—The structural integrity of the expressed segments of p115 RhoGEF was assessed by measuring their ability to associate with RhoA. At the highest concentration of immobilized RhoA (500 pmol, 10 μM), all expressed segments of p115 RhoGEF, except those lacking the PH domain (p115 1–10, 4–10, 5–10, and 19–10), bound stably and to similar extents to RhoA (Fig. 3). The relative affinity of these p115 pieces for RhoA was assessed by comparing their interaction with lower amounts (20 and 50 pmol) of immobilized GST-RhoA (Fig. 3). Interestingly, pieces...
Regulation of p115 RhoGEF

The apparent turnover rate for each p115 RhoGEF construct was expressed as moles of RhoA bound to GTP·S/min/mol GEF or as the number of GTP·S molecules that bind to RhoA per min in the presence of one molecule of exchange factor. This was determined by measuring the amount of RhoA that bound [35S]GTP·S over time and at multiple concentrations of exchange factor as described under “Materials and Methods.” The rates from each time course were plotted against the amount of exchange factor used, and an average apparent turnover rate was determined by linear regression analysis. The coefficient of determination, $R^2$, measures the degree to which the data fit a linear regression model. The last column expresses the activity of each construct relative to the activity of the full-length enzyme. For a description of the constructs see Fig. 1A.

### Table I

| p115 RhoGEF constructs | Rate of turnover $min^{-1}$ | $R^2$ value | Fraction of full-length activity |
|------------------------|-----------------------------|-------------|---------------------------------|
| p115 full-length        | 140                         | 0.99        | 1.0                             |
| p115-(3–14)             | 60                          | 0.99        | 0.4                             |
| p115-(ANH₂)             | 60                          | 0.99        | 0.4                             |
| p115-(252–288)          | 34                          | 0.99        | 0.2                             |
| p115-(COCOOH)           | 14                          | 0.99        | 0.09                            |
| p115-(19–11)            | 12                          | 0.99        | 0.09                            |
| p115-(4–11)             | 7                           | 0.99        | 0.05                            |
| p115-(4–14)             | 6                           | 0.99        | 0.04                            |
| p115-(1–10)             | 3                           | 0.99        | 0.02                            |
| p115-(19–10)            | 0.5                         | 0.99        | 0.003                           |
| p115-(4–10)             | 0.5                         | 0.99        | 0.003                           |
| p115-(5–10)             | 0.5                         | 0.99        | 0.003                           |

**FIG. 2.** Characterization of antibodies directed against p115 RhoGEF. A, specificity of the p115 RhoGEF antibodies generated for this study. Lysates were prepared from 35-mm plates of COS cells that had been transfected with vectors (1 µg of DNA) for expression of Myc-tagged full-length p115 RhoGEF (p115 FL), the N-terminal p115 RhoGEF-(1–18) (RGS), p115 RhoGEF-(4–10) (DH), or the C-terminal p115 RhoGEF-(16–14) (C-Term.). After separation by SDS-PAGE, samples were subjected to immunoblot analysis with either U2760 (1:4000 dilution), U2762 (1:3000 dilution), or U2764 (1:3000 dilution) as indicated and described under “Materials and Methods.” B, detection of endogenous p115 RhoGEF. Lanes contained the following: A, 15 µg of lysate from COS cells transfected with the pCMV5-myc vector expressing p115 RhoGEF; B, 15 µg of protein from a lysate derived from COS cells transfected with control vector; C, 10 ng of purified full-length p115 RhoGEF protein. Samples were separated by SDS-PAGE and subjected to immunoblot analysis with U2760, U2762, or U2764 as indicated under “Materials and Methods.”

**FIG. 3.** Binding of p115 RhoGEF proteins to immobilized GST-tagged RhoA. Purified p115 RhoGEF constructs (5 pmol) were incubated with 0, 20, 50, or 500 pmol of immobilized GST-RhoA as described under “Materials and Methods.” The unbound fractions (F) and the bound fractions (B) that were eluted from the beads were subjected to SDS-PAGE and analyzed by immunoblotting (IB) with the U2762 antibody (1:3000 dilution).

lacking amino acids 1–252 of p115 RhoGEF (p115 RhoGEF ANH₂, 4–14, 4–11, and 19–11) had a higher affinity for RhoA. The similar extents of association of the pieces, which bind at high concentrations of RhoA, indicate that the preparations possessed equivalent stoichiometries of RhoA-binding sites and thus provide an assessment of proper folding. Therefore, any differences in affinity were due to deletion of the domain rather than the result of gross misfolding. The structural integrity of constructs lacking the PH domain could not be demonstrated by this assay, presumably due to their relatively low affinity for RhoA that did not provide stable binding with the pieces under these conditions.

**Activation of RhoA by p115 RhoGEF**—The ability of the various constructs of p115 RhoGEF to stimulate binding of GTP·S to RhoA was measured over time and at multiple concentrations of each form of the exchange factors. Apparent turnover numbers were then determined from plots of the initial rates of the binding reactions in order to determine the initial rates of stimulation (Table I). Under the conditions utilized, full-length p115 RhoGEF is the most active enzyme and can stimulate ~140 RhoA molecules to bind GTP·S every minute. Deletion of either N-terminal or C-terminal portions of the enzyme resulted in reduced nucleotide exchange activity (Table I). To map the N-terminal region of p115 RhoGEF responsible for affecting exchange activity, a set of serial deletions was examined. Removal of either the first 50 amino acids (p115 RhoGEF-(3–14)) or the first 248 amino acids (p115 RhoGEF-(ANH₂)) of the enzyme reduced activity modestly by about 50%. In contrast, removal of the first 288 amino acids (p115 RhoGEF-(4–14)) reduced activity to only 4% of wild type enzyme. A significant but less dramatic loss in activity to 24% of wild type was observed when only amino acids 252–280 were deleted internally (p115 RhoGEF-(Δ252–280)).

Removal of the C-terminal 152 amino acids (p115 RhoGEF-(COCOOH)) reduces activity of the enzyme to about 10% of wild type activity and largely negated the effect of removing the N terminus. When deletions of amino acids 1–248 or 1–288 were assessed in mutants that also lack the C-terminal 760–912 amino acids (p115 RhoGEF-(19–11) and p115 RhoGEF-(4–11), respectively), there was either no effect or a modest 50% reduction in RhoA exchange activity compared with deletion of the C terminus alone. Alternatively, the C-terminal deletion still reduced activity when applied to the shorter N-terminal truncation (Δ1–248) but had no further effect when added to the larger N-terminal truncation (Δ1–288). These results suggest
an interactive dependence of the N and C termini with respect to expression of activity and point toward a dominant role for an area including amino acids 249–288.

Further removal of the PH domain from the C terminus (p115 RhoGEF-(1–10)) severely reduced activity to 2% of the wild type enzyme. Whereas deletions from the N terminus could further reduce activity (p115 RhoGEF-(19–10), p115 RhoGEF-(4–10), and p115 RhoGEF-(5–10)), there no longer appears to be an independent role for the internal amino acids 249–288. The smallest piece of p115 RhoGEF assessed (p115 RhoGEF-(19–11)) was composed of only the homologous DH domain. The presence of exchange activity confirmed the predicted enzymatic capacity of this region, but the low rate of turnover also indicates the efficacious effects of the other regions of the protein on this activity.

**Intracellular Localization of p115 RhoGEF**—The intracellular localization of endogenous p115 RhoGEF was examined by cellular fractionation. Cytosolic and membrane fractions from both NIH3T3 and COS cells were separated by SDS-PAGE and immunoblotted with the U2764 antiserum (1:4000 dilution). Purified p115 RhoGEF-(1–10) was also run as the standard. The domains of p115 RhoGEF responsible for membrane localization of overexpressed protein were studied by transfection of the different Myc-tagged p115 RhoGEF pieces in COS cells (Fig. 4B). Equivalent aliquots of membrane and cytosolic fractions (20% of the total) were subjected to immunoblot analysis using an antibody directed against the Myc tag. Express wild type p115 RhoGEF was distributed abundantly between both membrane and cytosolic fractions. This was also the case for the truncated p115 RhoGEF-(1–10) (aa 1–637), p115 RhoGEF-(19–11) (aa 246–760), p115 RhoGEF-(4–11) (aa 288–760), and the expressed RGS domain alone (amino acids 1–252). In contrast, a piece containing the DH domain alone, p115 RhoGEF-(19–10) (amino acids 248–637), was found almost entirely in the cytosolic fraction. This suggests that either the RGS or the PH domain can account, respectively, for the avidity of p115 RhoGEF-(1–10) or p115 RhoGEF-(19–11) and -(4–11) for the membrane fraction. Interestingly, the presumed ability of the PH domain to effect association with the membrane fraction is attenuated by the presence of the C terminus; thus p115 RhoGEF-(4–14) (aa 288–912) was found almost entirely in the cytosol.

**In Vivo Activity of Recombinant p115 RhoGEF**—The activation of RhoA by p115 RhoGEF pieces in vivo was assessed by two methods. An indirect assay utilized co-expression of a reporter plasmid that contained the firefly luciferase gene under the control of a mutated serum response element (SRE.L). Transcriptional stimulation by this element in response to activation of RhoA has been well documented (22, 30). In the current experiments, co-transfection of a plasmid expressing the botulinum C3 exoenzyme was used to block signaling by RhoA. Attenuation of transcription by expression of the C3 exoenzyme in response to p115 RhoGEF pieces indicates that the exchange factors were acting through RhoA (Fig. 5C). Selective regulation was indicated by the failure of p115 RhoGEF pieces to affect transcription regulated by the response element for NF-κB (data not shown).

Expression of full-length p115 RhoGEF stimulated SRE.L-dependent transcription by a modest 2–3-fold (Fig. 5A). All p115 RhoGEF pieces, which retained the C-terminal 152 amino acids (p115 RhoGEFs (ΔNH2) and p115 RhoGEF-(4–14)), produced similar modest effects (<4-fold activation of SRE.L). However, p115 RhoGEF pieces that contained the DH/PH domains, but lacked the C terminus (p115 RhoGEF (ΔCOOH) and p115 RhoGEF-(4–11)), elicited much more efficacious stimulation (>10-fold) of luciferase transcription (Fig. 5A). This stimulatory effect required the PH domain (contrast the basal efficacy of 1–10 in Fig. 5B with ΔCOOH in A). Since the p115 RhoGEF pieces (except p115 RhoGEF-(4–11)) expressed to similar extents in COS cells (Fig. 4B) and HEK293 cells (data not shown), it is most likely that differences in the specific activity of the p115 RhoGEF pieces caused the differences in activation of SRE.L. Pathways dependent on RhoA can also be stimulated by LPA in these cells. Whereas LPA could stimulate the expression of luciferase, there was not an apparent regulation of overexpressed p115 RhoGEF pieces, which either contained or lacked the RGS domain (Fig. 5B).

Activation of RhoA was also assessed more directly by co-transfection of HEK293 cells with HA-tagged RhoA and p115 RhoGEF constructs, which were then probed with GST-RBD for RhoA that contained bound GTP (26). The ratio of RhoA isolated with GST-RBD to total RhoA in the cell is a measure of the activated state of this GTPase. p115 RhoGEF-(19–11), which lacks the RGS and C-terminal regions, increased cellular HA-RhoA (GTP) much more potently than the full-length exchange factor (Fig. 6A and B). However, as the levels of expression of both p115 RhoGEF proteins increased, the efficacy of full-length p115 RhoGEF began to approach that of the truncated p115 RhoGEF-(19–11). This would be consistent with the existence of an intracellular inhibitor that bound to the C terminus of p115 RhoGEF but existed in limiting quantity.

**Activity Measurements of Endogenous p115 RhoGEF**—Because p115 RhoGEF stimulates RhoA to bind GTP, but is itself
activated by membrane-associated Ga13, the dynamics of p115 RhoGEF movement between the membrane and cytosol were examined. p115 RhoGEF was almost entirely cytosolic in the resting state (Fig. 4A) but moved to the membrane in response to LPA or 10% FBS (Fig. 4B). A 5-min treatment of the NIH3T3 cells with LPA or 10% FBS increased the amount of activated RhoA 7- to 4-fold, respectively. By 10 min, the fraction of active RhoA decreased to only 2-fold above untreated cells. Similar results were observed with the HEK293 cells.

Lysates that had been probed with GST-RBD were subsequently used to isolate endogenous p115 RhoGEF by immunoprecipitation with the polyclonal antibody, U2764 (Fig. 7D). The immunoprecipitated exchange factor was then tested in vitro for its ability to stimulate guanine nucleotide exchange on RhoA over time (Fig. 7C). Whereas similar amounts of immunoreactive p115 RhoGEF were isolated from all cells, greater exchange activity was observed in samples from cells treated with either LPA or sphingosine 1-phosphate. This suggests that a higher activity form of p115 RhoGEF is induced by the two stimuli and that increased activity of p115 RhoGEF is at least partially responsible for the activation of RhoA by LPA and S1P.

**DISCUSSION**

**Structural Requirements for Full Exchange Activity—Numerous proteins have been identified as exchange factors for the Rho subfamily of monomeric GTPases. Expression of truncated proteins and structural analysis have established that their homologous DH domains form the catalytic site for stimulation of Rho GTPases. However, the understanding of the actual catalytic mechanisms of these proteins and their regulation of activity is sparse. p115 RhoGEF is a particularly interesting model. It contains an RGS-like domain that is required for regulation of the exchange activity of GEF by Ga13 (17). In addition, comparison of new truncated forms of p115 RhoGEF identify regions of the enzyme that flank the DH and PH domains but have profound influence on the activity of the enzyme. A region of 37 amino acids lying between the RGS and DH domains reduced activity by more than 75–90% when deleted (p115 RhoGEF full length versus p115-(252–288) and p115-(4–14) in Table I).**

Deletion of the C terminus of the protein also reduced exchange activity by more than 75–90% when deleted (p115 RhoGEF full length versus p115-(4–11) or p115 RhoGEF full-length cDNA used in the transfection.)
FIG. 7. Regulation of endogenous p115 RhoGEF by extracellular stimuli. A, LPA promoted partial targeting of p115 RhoGEF from the cytosol to the membrane. Membrane and cytosolic fractions were prepared from serum-starved NIH3T3 cells that were either untreated (basal) or treated with 3 μM LPA for 5 min. Proteins were separated via SDS-PAGE, and p115 RhoGEF was visualized by immunoblot analysis using the U2764 antibody (1:4000 dilution). B, endogenous RhoA was activated within 5 min by serum or LPA. After overnight deprivation of serum, NIH3T3 and HEK293 cells were treated with serum-free DMEM containing 3 μM LPA for 5 min, DMEM containing 10% FBS for 5 min, or DMEM containing 10% FBS for 30 min, as indicated. The GTP-bound form of RhoA was precipitated with immobilized GST-RBD from lysates prepared from these cells, and the amount of activated RhoA was visualized by immunoblotting (top strip) as described under "Materials and Methods." Total RhoA in 20 μl of lysate was visualized by immunoblot analysis (lower strip) using the RhoA-specific antibody. C, activity of endogenous p115 RhoGEF isolated from cells treated with LPA or S1P. p115 RhoGEF was immunoprecipitated (using the U2764 antibody) from lysates derived from monolayers of serum-starved NIH3T3 cells that were either treated for 5 min with serum-free DMEM (closed circles), serum-free DMEM with 3 μM LPA (closed triangles), or serum-free DMEM with 1 μM S1P (open circles). Immunoprecipitates were divided equally and assayed for ability to stimulate nucleotide exchange on RhoA or used in D. D, equal amounts of the remaining portion of the immunoprecipitates not used for the RhoA exchange assays in C were separated by SDS-PAGE and immunoblotted for p115 RhoGEF with the U2764 antibody (1:4000 dilution) (as indicated).

Interestingly, losses of activity due to deletions in the RGS/DH linker region and the C termini were not additive when both were applied to the same protein. This suggests that an intramolecular or intermolecular association between these two regions of the enzyme may be required for expression of full activity by the DH domain. Preliminary analysis of mass by sedimentation equilibrium indicates that p115 RhoGEF associates with itself in tetrameric or larger complexes (data not shown); whether self-association occurs through interaction of N and C termini is not known.

Pieces of p115 RhoGEF that lack the N-terminal regions (4–14), (4–11), (19–11), and (∆NH2) bind more tightly to RhoA (Fig. 3), but display, respectively, only 4, 5, 9, and 40% of the activity of the full-length enzyme (Table I). This tight association with RhoA suggests that these reductions in activity may be due to a slower release of p115 RhoGEF from RhoA. The rate-limiting step for turnover of the Ras GTPase has been shown to be release of the GTPase from its exchange factor (32). If release of p115 RhoGEF from RhoA is also rate-limiting, then pieces missing the N-terminal regions may have lower activity due to their higher affinity for RhoA, which is devoid of bound nucleotide. In this case, GTP would be less able to displace the exchange factor and potentially slow the overall rate of cycling of the GEF.

The Role of the PH Domain in Vivo and in Vitro—The PH domain was initially identified as a region (~100 amino acids) that was duplicated in pleckstrin, the predominant substrate in platelets for phosphorylation by protein kinase C. Although the primary amino acid sequences of PH domains are quite divergent, they possess the same structural motif, occur in numerous signaling proteins, and presumably provide a limited array of functional characteristics. The most touted properties identified with specific PH domains are binding to phosphoinositides and targeting to membranes, although only about 10% of all characterized PH domains bind phosphoinositides with high affinity and specificity (33). The functions of the PH domains that invariably follow DH domains in exchange factors that act on members of the Rho family appear to be of broader impact and diversity. The PH domain of Vav binds phosphatidylinositol 4,5-bisphosphate, and this association inhibits the action of this exchange factor on Rac1 (34). Removal of the PH domain lowered the exchange activity of Trio on Rac1 to 1% of wild type activity (35). The crystal structure of the DH and PH domains that mediate exchange activity on Rac1 by Sos1 (Son of sevenless) reveals a PH domain that folds back toward the DH domain to form an L-like structure. When a GTPase is modeled into the binding surface of Sos1, the PH domain is positioned to interact directly with the monomeric GTPase and potentially influence function (36). In contrast, PH domains in other exchange factors do not appear to have a direct role in enzymatic activity. In the complex of Tiam1 with Rac1, the PH domain is oriented away from Rac1 where it would be unlikely to interact with the GTPase (37).

Both Dbl and Lfc require PH domains for membrane localization of the exchange factors, but deletion of their PH domains does not affect the intrinsic activity of the remaining exchange factor to stimulate nucleotide exchange (38, 39).
failure to observe an effect of phosphatidylinositol 4,5-bisphosphate on the activity of p115 RhoGEF in vitro suggests that binding of phosphoinositides is not involved (data not shown).

The RGS Domain of p115 RhoGEF Promotes Membrane Localization—A second region that promotes localization to the particulate fraction of cells is found within the N-terminal 252 amino acids containing the RGS domain. Proteins that contain this region but lack the PH domain (p115 RhoGEF-(1–18) and p115 RhoGEF-(1–10)) show substantial partitioning with the particulate fraction (Fig. 3). A possible mechanism for this localization could be acylation of a double cysteine motif at amino acids 77–78; however, we were unable to detect any palmitoylation of either endogenous or overexpressed p115 RhoGEF. Recruitment of p115 RhoGEF to the membrane by overexpressed Gα13 was reported recently (40). It is likely that Gα13 is at least partially responsible for targeting p115 RhoGEF through association with its N terminus. The functional relevance of localization by the N-terminal domain is suggested by a small but significant stimulation of RhoA by expression of p115 RhoGEF-(1–10) but not the complementary construct lacking the N terminus (p115 RhoGEF-(19–10)) (Fig. 5B).

Regulation of Recombinant p115 RhoGEF in Vivo—The dissimilarity between activities of p115 RhoGEF constructs when measured in vitro versus in vivo predicts the existence of regulatory processes that attenuate the activity of this protein. Purified, full-length p115 RhoGEF is more active than any of its deletion mutants in vitro (Table I) but less active than several of the mutants in vivo (Figs. 5 and 6). Thus, mutants of p115 RhoGEF that lack 152 amino acids of the C terminus (p115 RhoGEF-(4–11), -(19–11), and -(ΔCOOH)) displayed less than 10% of the exchange activity of the full-length enzyme in vitro (Table I) but were often over 5-fold more active than the full-length enzyme in stimulating RhoA when expressed in cells (Fig. 5 and 7). This contrasts with other exchange factors such as Dbl (11) and the Rho-selective p190 RhoGEF (31) that are highly active in vivo when expressed as the full-length proteins.

The high activity of purified p115 RhoGEF also suggests that there has to be a mechanism to maintain a low activity state in vivo. The relative increase in activity in vivo of p115 RhoGEF that lacked its C terminus would be explained by the existence of a factor that could attenuate the activity of the exchange factor by binding to or modifying its C terminus. If such a factor were limited in cells, it could be overwhelmed at sufficiently high concentrations of overexpressed exchange factor. Such a mechanism would explain results from experiments that show that p115 RhoGEF-(19–11) activated RhoA to a greater extent at low levels of expression than the full-length enzyme. As the level of p115 RhoGEF-(19–11) was increased, RhoA was increasingly activated in a relatively linear fashion. In contrast, varied amounts of full-length p115 RhoGEF showed little effect at lower levels of expression but displayed a steep increase in the rate of activation of RhoA at the highest concentration. At the highest levels of expressed p115 RhoGEF, the efficacies for activation of RhoA by either full-length exchange factor or truncated p115 RhoGEF-(19–11) were converging.

Association of overexpressed and endogenous p115 RhoGEF with the membrane fraction also suggests evidence for an intracellular inhibitor. The p115 RhoGEF pieces that lack the C-terminal 760–912 aa (and also lack the RGS domain) partition to the particulate fraction much better than pieces containing the C terminus (contrast p115 RhoGEF-(4–14) to -(19–11) or -(4–11) in Fig. 4B). The existence of endogenous p115 RhoGEF largely in the cytosol (Fig. 4A) as opposed to the equal distribution of overexpressed full-length p115 RhoGEF (Fig. 4B) suggests that some factor is keeping endogenous p115 RhoGEF in the cytosol. This factor would then be overwhelmed at high levels of protein expression and give rise to the greater proportion of overexpressed full-length p115 that resides in the membrane fraction.

Characterization of Endogenous p115 RhoGEF—Previous work involving p115 RhoGEF has relied on either in vitro assays using purified recombinant protein or overexpression of the protein in cells. Here we also examined the properties and behavior of endogenous p115 RhoGEF in response to stimuli. By comparative immunoblot analysis of p115 RhoGEF in lysates from NIH3T3 and COS cells and a known amount of purified p115 RhoGEF, it is estimated that p115 RhoGEF is ~0.01%–0.02% of the total cellular protein (Fig. 2B and Fig. 4A). Most endogenous exchange factor was found to partition in the cytosol (Fig. 4A) when cells were grown under resting conditions.

Stimulation of NIH3T3 and HEK293 cells with LPA resulted in activation of both endogenous RhoA (Fig. 7B) and p115 RhoGEF (Fig. 7C) within 5 min. Interestingly, the rapid targeting of p115 RhoGEF to the membrane within 5 min of LPA treatment (Fig. 7A) correlated temporally with previous reports for membrane targeting of RhoA (41, 42). This would place p115 RhoGEF in the locale of Gα13 and suggests that p115 RhoGEF may act on RhoA that is also targeted to the membrane. HEK293 cells, which have a higher basal level of RhoA activity (Fig. 7B), also display a higher concentration of endogenous p115 RhoGEF protein (data not shown). These correlations support a role for p115 RhoGEF as a major activator of RhoA in vivo.

It is of interest that the activation of p115 RhoGEF can be measured after immunoprecipitation. Whereas Gα13 is known to activate p115 RhoGEF, it is unlikely to be responsible for the elevated activities of the immunoprecipitated protein because Gα13 was undetectable in immunoprecipitates by Western blot analysis (data not shown). Additionally, exchange assays were performed several hours after cells were lysed, which is far longer than the half-life for hydrolysis of GTP bound to Gα13 (27). Phosphorylation is also an unlikely explanation for this activation. Whereas endogenous p115 RhoGEF was found to be phosphorylated, the level of phosphorylation was not discernibly altered in response to LPA, S1P, or 10% FBS (data not shown). It is important to note that purified recombinant p115 RhoGEF is very active in the absence of any activators (Table I), but modest expression of recombinant full-length p115 RhoGEF provides only small effects on the intracellular activity of RhoA (Figs. 5 and 7). One speculation is that an unidentified inhibitor is co-immunoprecipitated in the lysates from serum-starved cells but is partially absent from immunoprecipitates of LPA-treated cells. Alternatively, an unknown post-translational modification that is removed or added in response to stimulation by LPA or S1P could account for the change in activity. The robust in vivo activity of p115 RhoGEF mutants that lack the C-terminal 152 amino acids of the protein strongly suggests that this region is a likely location for mediation of this regulation. Such an event would provide a second regulatory pathway for modulation of activity by G protein-coupled receptors. This second regulatory mechanism could work independently or in concert with stimulation induced by the G13p protein.

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Identification of Potential Mechanisms for Regulation of p115 RhoGEF through Analysis of Endogenous and Mutant Forms of the Exchange Factor
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