A Novel PDZ Domain Containing Guanine Nucleotide Exchange Factor Links Heterotrimeric G Proteins to Rho*

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Small GTP-binding proteins of the Rho family play a critical role in signal transduction. However, there is still very limited information on how they are activated by cell surface receptors. Here, we used a consensus sequence for Dbl domains of Rho guanine nucleotide exchange factors (GEFs) to search DNA data bases, and identified a novel human GEF for Rho-related GTPases harboring structural features indicative of its possible regulatory mechanism(s). This protein contained a tandem DH/PH domain closely related to those of Rho-specific GEFs, a PDZ domain, a proline-rich domain, and an area of homology to Lsc, p115-RhoGEF, and a Drosophila RhoGEF that was termed Lsc-homology (LH) domain. This novel molecule, designated PDZ-RhoGEF, activated biological and biochemical pathways specific for Rho, and activation of these pathways required an intact DH and PH domain. However, the PDZ domain was dispensable for these functions, and mutants lacking the LH domain were more active, suggesting a negative regulatory role for the LH domain. A search for additional molecules exhibiting an LH domain revealed a limited homology with the catalytic region of a newly identified GTPase-activating protein for heterotrimeric G proteins, RGS14. This prompted us to investigate whether PDZ-RhoGEF could interact with representative members of each G protein family. We found that PDZ-RhoGEF was able to form, in vivo, stable complexes with two members of the Go12 family, Go13, and that this interaction was mediated by the LH domain. Furthermore, we obtained evidence to suggest that PDZ-RhoGEF mediates the activation of Rho by Go13 and Go12. Together, these findings suggest the existence of a novel mechanism whereby the large family of cell surface receptors that transmit signals through heterotrimeric G proteins activate Rho-dependent pathways: by stimulating the activity of members of the Go12 family which, in turn, activate an exchange factor acting on Rho.

The Ras superfamily of GTPases comprises approximately 50 members that can be divided into several families, Ras, Rho, Sar, Rab, Arf, and Ran, based on their primary sequence as well as on their cellular activities (1–3). Whereas the Rab, Arf, and Sar groups participate in the transport of proteins and vesicles among different intracellular compartments, the Ran proteins function in nuclear transport, and Ras plays a central role in cell proliferation and differentiation (2, 4). In the case of Ras, recent studies have revealed how it works at the molecular level. This small GTP-binding protein exchanges GDP for GTP upon activation of Ras-specific guanine nucleotide exchange factors (GEFs)1 (5), and in the GTP-bound state, Ras physically associates with Raf (6) thereby recruiting this serine threonine kinase to the plasma membrane. This causes the activation of Raf and initiates the activity of a sequential cascade of kinases leading to the stimulation of mitogen-activated protein kinases (MAPKs), p42MAPK and p44MAPK, also known as extracellular signal-regulated kinases-2 and -1, respectively, which, in turn, control the activity of nuclear transcription factors that are critical for cell growth (7, 8).

The Rho family of GTP-binding proteins, which consists of the Rho, Rac, and Cdc42 subfamilies, has been shown to regulate several aspects of cytoskeleton function (4). For example, Rho participates in the formation of actin stress fibers and mediates the redistribution of cytoskeletal components (4, 9). Rac is involved in the regulation of lamellipodia (pleat-shaped protrusions at the cell periphery) and membrane ruffling (10); and Cdc42 regulates the formation of thin finger-like cytoplasmic extensions known as filopodia (11). These proteins play an important role in the regulation of cell morphology, cell aggregation, tissue polarity, cytokinesis, cell motility, and also in smooth muscle contraction (12–14). However, recent evidence suggests that Rho proteins are also integral components of signaling pathways leading to transcriptional control. For example, Rac and Cdc42 regulate the activity of the c-Jun amino-terminal kinase (JNK) thereby affecting the transcriptional activity of c-Jun (15), and Rho has been recently shown to induce expression from the serum responsive element (SRE) through the transcriptional activation of the serum response factor (SRF) (16).

The functional activity of small GTP-binding proteins of the Ras superfamily is tightly regulated in vivo by proteins that control their GDP/GTP bound state. Whereas GEFs promote the exchange of GDP for GTP thus activating Ras-like proteins (2), GTPase-activating proteins increase the low intrinsic rate of GTP hydrolysis of small GTPases (reviewed in Ref. 3) and are negative modulators. The mechanisms of activation of GEFs for Ras by cell surface receptors have been intensively investigated. For example, the biochemical route connecting the epidermal growth factor-receptor tyrosine kinase to Ras has been recently identified (5, 17), and includes the phosphorylation of the receptor itself on tyrosine residues, thus creat-

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1 The abbreviations used are: GEF, guanine nucleotide exchange factor; LH domain, Lsc homology domain; MAPK, mitogen-activated protein kinase; JNK, c-Jun amino-terminal kinase; SRE, serum response element; SRF, serum response factor; GAP, GTPase-activating protein; DH domain, Dbl homology domain; PH domain, pleckstrin-homology domain; SH, Src homology; RGS, regulators of G protein signaling; CAT, chloramphenicol acetyltransferase.

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Many GEFs for Rho, Rac, and Cdc42, including dbl, ost, lfc, lbc, vav, ect2, tim, and net (reviewed in Refs. 18 and 19) were discovered by virtue of their ability to transform NIH 3T3 cells when overexpressed or when activated by trancers.

Many GEFs for Rho-like proteins, the protein product of the vav proto-oncogene, proto-Vav, also exhibits a Src homology (SH) 2 domain flanked by two SH3 domains (22, 23), and we have recently shown that tyrosine phosphorylation of proto-Vav by hematopoietic specific tyrosine kinases can activate its GEF activity for Rac both in vitro and in vivo (24, 25). However, no other GEF for Rho-like proteins has been found to be regulated by tyrosine phosphorylation, nor to contain a phosphotyrosine-binding domain such as an SH2 or PTB domains. Furthermore, the vast majority of the known GEFs for small GTPase-binding proteins of the Rho family are expressed in a very restricted tissue-specific manner (18, 19), and their mechanism of activation is still largely unknown.

In this study, we explored the existence of novel GEFs for Rho-like proteins possessing structural domains that might suggest a role in signal transduction. Here, we report the identification of a novel, ubiquitously expressed GEF for Rho-like proteins containing a PDZ domain. This protein, termed PDZ-RhoGEF, was found to activate biochemical pathways specific for Rho, in a Rho-dependent manner. Interestingly, PDZ-RhoGEF was found to be closely related to the *Drosophila* DRhoGEF2, and recent genetic analysis suggests that DRhoGEF2 acts downstream of the concertina gene, a *Drosophila* Ga12 homolog. Here, we found that PDZ-RhoGEF physically associates in vivo with activated α subunits of heterotrimeric G proteins of the Ga12 family, Ga12α and Ga12β. Association was found to occur through a novel structural domain, termed Lac homology (LH) domain, located between the PDZ and the DH domain, and also present in the NH2-terminal, regulatory domain of the *lac* proto-oncogene product and its human homolog, p115-RhoGEF. This LH domain is distantly related to the G protein-binding region of a family of proteins known as regulators of G protein signaling (RGSs) (26). Together, our present findings suggest the existence of a novel pathway by which the large family of G protein-coupled receptors communicates to Rho through the activation of Ga12α/Ga12β and the physical association between Ga12α or Ga12β with LH containing GEFs for Rho, thereby stimulating Rho-dependent pathways.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids—**KIAA0380 (human PDZ-RhoGEF), kindly provided by T. Nagase, Kazusa DNA Research Institute, Japan, was subcloned into the pcEF vector as an Asp718-NolI fragment, thus generating the pCEF-PDZ-RhoGEF expression plasmid. Then, the coding sequence for the AU1 hexapeptide (DTYRYI) was cloned in-frame with the open reading frame of PDZ-RhoGEF, immediately upstream of the termination codon, thus generating a carboxyl-terminal AU1-tagged PDZ-RhoGEF. CDNAs encoding deletion mutants of PDZ-RhoGEF, as indicated in the corresponding figures, were generated by restriction enzyme digestion or polymerase chain reaction amplification using pCEF-PDZ-RhoGEF-AU1 as a template. Sequences of mutagenic oligonucleotides will be made available upon request.

Plasmids expressing epitope-tagged MAPK and JNK, pcDNA3 HA-MAPK and pcDNA3 HA-JNK, respectively, as well as expression plasmids for constitutively activated forms of Ras, RhoA, Ga12α, Ga12β, Ga12γ, Ga13γ, β1, and γ subunits of G proteins and RhoI41A, a RhoA mutant insensitive to the C3 toxin activity, were described previously (15, 27). Reporter plasmids that express the chloramphenicol acetyltransferase (CAT) gene under the control of the wild-type serum response element (SREwt), or a mutant l-fos promoter (SREmutL), or a mutant lacking the ternary complex-factor-binding site (SREmutL) as well as an expression vector for the C3 toxin were kindly provided by R. Treisman (16).

**Northern Blot Analysis—**Human multiple tissue Northern blots, each lane containing 2 μg of poly(A)+ RNA, were purchased from Clontech. Total RNA was isolated from several cell lines by a disposable kit (QIAGEN) according to the manufacturer's instructions, then separated by electrophoresis on a 2% denaturing formaldehyde-agarose gel (20 μg of RNA/lane), and transferred to HybondTM-N nylon membrane (Amersham Life Science). The CDNA probe used for analysis of the PDZ-RhoGEF mRNA was prepared using as a template a 1832-base pair SacI-XbaI fragment derived from pCEF-PDZ-RhoGEF, containing both 3'-translated and -untranslated regions of PDZ-RhoGEF cDNA. Human β-actin cDNA (2.0 kilobase pairs) was also used as a control probe. Probes were labeled using a Random Primer DNA labeling kit (Boehringer-Mannheim) with [α-32P]dCTP, and RNA hybridization performed as described (28).

**Cell Lines and Transfection—**Human kidney 293T cells and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. NIH 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. 293T cells were transfected by the calcium-phosphate precipitation technique to examine the expression of PDZ-RhoGEF and its mutants or using LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the manufacturer's protocol for the co-immunoprecipitation study. Transfection into NIH 3T3 cells were carried out by the calcium-phosphate precipitation technique, and COS-7 cells were transfected by the DEAE-dextran method. In each experiment, total amount of DNA was adjusted to 3–10 μg/plate with vector alone without insert. For the transfection into 293T cells, tissue culture plates were treated with phosphate-buffered saline containing 20 μg/ml poly-γ-lysine for 10 min before seeding the cells, to prevent them from detaching from the plates.

**Kinase Assays—**MAPK activity in cells transfected with an epitope-tagged MAPK (HA-ERK2, referred in here as HA-MAPK) was determined as described previously (15), using myelin basic protein (Sigma) as a substrate. JNK assays in cells transfected with an epitope-tagged JNK (HA-JNK) was also determined as described previously (15), using myelin basic protein, bacterially expressed GST-ATF2(96) fusion protein as a substrate. Samples were separated by SDS-gel electrophoresis on 12% acrylamide gels. Autoradiography was performed with the aid of an intensifying screen. Parallel lysates of cells transfected with the HA-MAPK or HA-JNK expression plasmids were processed for Western blot analysis using an antibody against the HA epitope.

**Reporter Gene Assays—**NIH 3T3 cells were transfected with different expression plasmids together with 1.0 μg of pCMV-β-gal, a plasmid expressing the enzyme β-galactosidase, and 1.0 μg of pSREmutL, the reporter plasmid expressing the CAT gene. 293T cells were transfected with expression vectors for PDZ-RhoGEF or its mutants together with 0.5 μg of pCMV-β-gal and 0.5 μg of pSREwt, the reporter plasmid expressing a luciferase gene under the control of the wild-type SRE. After overnight incubation, NIH 3T3 cells and 293T cells were washed twice with phosphate-buffered saline, and kept for approximately 24 h in Dulbecco's modified Eagle's medium supplemented with 0.5% calf serum or 0.5% fetal bovine serum, respectively. Cells were then lysed using reporter lysis buffer (Promega). Additional DNAs were added to the transfection mixtures as indicated in each figure. CAT activity was measured in the cell extracts by incubation at 37 °C for 10–16 h in the presence of 0.25 μCi of [14C]chloramphenicol (100 mCi/mmol) (ICN) and 200 μg/ml butyryl-CoA (Sigma) in 0.25 % Tris-HCl, pH 7.4. Labeled butyryl products were extracted using a mixture of Xylenes (Aldrich) and counted as described (15). Luciferase activity in cell extracts was measured using Luciferase assay system (Promega). β-Galactosidase activity present in each sample was assayed by a colorimetric method, and used to normalize for transfection efficiency.

**Co-immunoprecipitation and Western Blot Analysis—**To confirm the expression of PDZ-RhoGEF and its mutants, 293T cells were transfected with vector or expression vector for each PDZ-RhoGEF-AU1 DNA construct. Then, cells were cultured for 48 h, washed twice with phosphate-buffered saline, and lysed at 4 °C in a buffer containing 25 mM HEPS, 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, and washing buffer.
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PDZ domain

PDZ-RhoGEF

DH domain

PH domain

Fig. 1. PDZ-RhoGEF contains several domains involved in signal transduction and is widely expressed in many human tissues. Amino acid sequence of PDZ-RhoGEF is depicted using the expected translational product of PDZ-RhoGEF, accession number AB002378, with several domains involved in signal transduction and widely expressed in many human tissues.
This search revealed the existence of a number of yet uncharacterized proteins exhibiting DH-like domains (data not shown). Subsequent analysis of their DNA sequences and their expected translatational products suggested that many of them encode putative GEFs for Rho-like proteins. One of them, KIAA0380, accession AB002378, was of particular interest and was further characterized. The corresponding plasmid DNA was obtained from Dr. T. Nagase, Kazusa DNA Research Institute, Japan, and its nucleotide sequence confirmed. Of interest, its open reading frame encodes a protein of 1522 amino acids, possessing areas of high homology to other signaling molecules (Fig. 1A). As depicted in Fig. 1B, this molecule contains a tandem of DH and PH domains, being the DH domain closely related to those of p115-RhoGEF (30) (53% identity, 74% similarity) and Lsc (31) (53% identity, 72% similarity), and to the recently identified DRhoGef2 (32, 33) (39% identity, 64% similarity). Furthermore, this molecule displays 35% of identity and 45% homology when analyzed for global homology with p115-RhoGEF/Lsc. However, the DH and PH domains were more distantly related to the DH domain of Db1 (20) and PH domain of pleckstrin (34), respectively. This molecule also contains an NH2-terminal region exhibiting extensive homology to a recently identified structural domain termed PDZ (35), which is involved in protein-protein interactions, and is also present in DRhoGef2. As discussed above, the DH domain is believed to be responsible for the nucleotide exchange activity of GEFs, and both p115-RhoGEF and Lsc have been shown to behave as Rho-specific guanine nucleotide releasing factors (30, 36). Thus, the newly identified molecule, which was tentatively named PDZ-RhoGEF, might represent a novel exchange factor for Rho.

PDZ-RhoGEF exhibits additional structural features, a proline-rich region (amino acids 149 to 160) and another area (amino acid 290–486) showing a high degree of homology (~34% identity and ~53% similarity) to Lsc, p115-RhoGEF, and DRhoGef2 (Fig. 1B), that was termed Lsc homology (LH) domain. Thus, PDZ-RhoGef exhibits a number of characteristics that suggest a function in signal transduction: a DH-PH domain highly related to those of Rho GEFs, a DH domain, and an area of homology to three other GEFs.

**PDZ-RhoGEF Is Widely Expressed—** The majority of the known GEFs for small GTP-binding proteins of the Rho family are expressed in a restricted tissue-specific manner. To investigate the pattern of expression of PDZ-RhoGEF, we performed Northern blot analysis of RNAs from a broad range of human tissues using a non-conserved region including both 3′-translated and -untranslated sequences, nucleotides 3959–5790, of the PDZ-RhoGEF cDNA as a probe. As shown in Fig. 1C, a prominent RNA transcript of approximately 7 kilobases was readily detected in many human tissues, albeit to a different extent. PDZ-RhoGEF is highly expressed in the brain, testis, heart, ovary, and placenta, and to lower levels in kidney, pancreas, spleen, prostate, colon, skeletal muscle, lung, and liver. Whereas no expression was detected in the thymus and small intestine, two RNA species were expressed at comparable levels in peripheral blood leukocytes. The nature of the smaller transcripts detected in leukocytes, as well as that of the additional minor RNA species detected in placenta, spleen, brain, and heart is still unknown, and under current investigation. Similarly, transcripts for PDZ-RhoGEF were detectable in fre-
FIG. 2. Activation of the SRE, but not MAPK cascades, by PDZ-RhoGEF. A, structures and expression of epitope-tagged wild type and NH₂-terminal truncated mutants of PDZ-RhoGEF. On the left panel, structure of the proteins encoded by each expression plasmid: Δ-127, Δ-238, Δ-702, and Δ-956 constructs code for amino acid residues 127 to 1522, 238 to 1522, 702 to 1522, and 956 to 1522 of PDZ-RhoGEF, respectively. On the right panel, lysates from cells transfected with the vector control or with expression plasmid carrying epitope-tagged forms...
quently used mammalian cell lines, such as HeLa, 293T, COS-7, and normal human keratinocytes, but limited expression was observed in NIH 3T3 cells (data not shown). Thus, we can conclude that PDZ-RhoGEF is expressed in a large variety of human tissues, rather than in a tissue-restricted manner.

Expression of Wild-type and Truncated Mutants of PDZ-RhoGEF—To begin exploring the biochemical specificity of PDZ-RhoGEF and the relative contribution of each structural domain, we engineered expression plasmids for epitope-tagged forms of wild-type and truncated PDZ-RhoGEF mutants. Initially, PDZ-RhoGEF was subcloned in an expression vector, pCEFL (37), and then the coding sequence for the hexapeptide DTYRI was cloned in-frame with the open reading frame of PDZ-RhoGEF immediately upstream of the termination codon, thus generating a carboxyl-terminal AU1-tagged PDZ-RhoGEF. As shown in Fig. 2A, when transfected into 293T cells, wild-type PDZ-RhoGEF was readily detectable with an anti-AU1 epitope-specific antibody. Similarly, each sequential NH2-terminal deletion mutant, Δ-127, Δ-238, Δ-702, and Δ-956, lacking progressively, the PDZ domain, the proline-rich region, the LH domain, and the DH domain, as depicted in Fig. 2A, were also detected in transfected 293T cells.

PDZ-RhoGEF Fails to Induce MAPK Cascades—Whereas Ras controls the MAPK cascade, recent data suggest that Rac and Cdc42 can regulate the activity of the JNK pathway (15). Thus, as an approach to investigate whether PDZ-RhoGEF activates small GTPases of the Ras, Rac, or Cdc42 class, we investigated the ability of PDZ-RhoGEF and its deletion mutants to activate MAPK and JNK in COS-7 cells. As shown in Fig. 2B, none of the PDZ-RhoGEF expression plasmids enhanced the activity of co-transfected HA-MAPK or HA-JNK, although, 12-O-tetradecanoylphorbol-13-acetate addition and activated Ras potently stimulated MAPK, and anisomycin and activated Dbl strongly activated JNK when used as controls under identical experimental conditions. Thus, these data strongly suggest that PDZ-RhoGEF cannot activate Ras and Rac/Cdc42 regulated pathways when expressed in COS-7 cells.

PDZ-RhoGEF Activates SRE in a Rho-dependent Manner—Recently, Rho proteins have been shown to signal to the SRE through a pathway affecting the transcriptional activity of SRF, independent of any MAPK described to date (16). Thus, we next asked whether PDZ-RhoGEF could induce expression from a reporter plasmid containing a mutated SRE which eliminated the ternary complex factor-binding site and which was shown to be potently activated by Rho (16). As shown in Fig. 2C, an activated form of Rho potently induced expression from this reporter system when used as a control and, under identical experimental conditions, PDZ-RhoGEF caused a remarkable, nearly 15-fold, elevation in CAT activity. Furthermore, whereas deletion of the PDZ and the proline-rich domain did not have any demonstrable effect, deletion of the entire NH2-termino-

![Fig. 3. Involvement of functional Rho proteins in the SRE activation by PDZ-RhoGEF.](image_url)

Figure 3: Involvement of functional Rho proteins in the SRE activation by PDZ-RhoGEF. NIH 3T3 cells were co-transfected with pSREmutL (1 μg/plate) and pCMV-β-gal (1 μg/plate) plasmid DNAs and expression vectors for the activated mutant of PDZ-RhoGEF (2 μg/plate, Δ-702), the C3 toxin, and the C3-insensitive mutant of RhoA (5 μg/plate, RhoA141), as indicated. 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 mean ± S.E. of triplicate samples from a typical experiment. Similar results were obtained in three independent experiments.
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Madin-Darby canine kidney cells, a typical Rho-dependent effect (data not shown). Collectively, these data indicate that PDZ-RhoGEF can effectively activate Rho-dependent pathways.

PDZ-RhoGEF Associates Physically with Heterotrimeric G Protein α Subunits of the Ga12 Family—A genetic screen for components of the Rho signaling pathway in Drosophila led to the identification of DRhoGEF2, the Drosophila homolog of PDZ-RhoGEF, as an essential molecule for directing cell shape changes associated with gastrulation during early embryo development (32, 33). Of interest, two other molecules were previously identified as critical for gastrulation, folded gastrulation receptor, concertina, a G protein α subunit related to Ga12 (41). Although a direct link between the folded gastrulation receptor, concertina and DRhoGEF2 is yet to be established, these studies suggested that this Rho-GEF might act downstream from heterotrimeric G proteins. Furthermore, a computer assisted search for proteins sharing areas of homology to the LH domain of PDZ-RhoGEF revealed that this domain exhibits limited similarity to a region within the putative catalytic domain of RGS14 (26), a newly discovered RGS for G protein α subunits (Fig. 4). Together, these findings prompted us to explore whether PDZ-RhoGEF could interact directly with a heterotrimeric G proteins.

As an approach, we co-expressed in 293T cells the AU1-tagged full-length PDZ-RhoGEF together with β1γ2 dimers or activated forms of representative members of each G protein α subunit family, Gaαq, Gaαq, and Gaαq, and Gaαq, in 293T cells. In each case, we used Gaαq proteins rendered constitutively active by replacing a critical glutamine residue within the C3 region that is essential for GTP hydrolysis for leucine (QL mutants) (1). Detailed biochemical characterization of each G protein subunit has been previously reported in our laboratory (29, 42–45). As shown in Fig. 5A, the tagged PDZ-RhoGEF and all transfected G protein α subunits and βγ heterodimers were expressed, as judged by Western blot analysis using anti-epitope monoclonal antibodies and G protein subtype-specific antisera. Surprisingly, we found that when PDZ-RhoGEF was immunoprecipitated, both members of the Gaαq family, Gaαq, and Gaαq, co-immunoprecipitated with this putative nucleotide exchange factor for Rho. No other G protein subunit was found to co-immunoprecipitate with PDZ-RhoGEF, nor were Gaαq and Gaαq, detectable in immunoprecipitates from control samples (Fig. 5A). To confirm these findings, we co-expressed the AU1-tagged PDZ-RhoGEF with NH2-terminal HA-tagged forms of Gaαq and Gaαq, and performed anti-AU1 and anti-HA Western blots on both anti-HA and anti-AU1 immunoprecipitates. As shown in Fig. 5B, the activated forms of Gaαq efficiently co-immunoprecipitated PDZ-RhoGEF. Similar results were obtained with Gaαq, although the co-immunoprecipitation of PDZ-RhoGEF was less efficient because the HA-tagged Gaαq was poorly expressed (data not shown). We concluded that both members of the Gaαq family physically associate in vivo with PDZ-RhoGEF, thus providing a direct link between heterotrimeric G proteins and small GTP-binding proteins of the Ras superfamily.

PDZ-RhoGEF Binds Heterotrimeric G Proteins through a Novel Structural Domain Designated LH—To investigate further the identity of the structural domains of PDZ-RhoGEF involved in binding to Ga12 and Ga13, we used the progressive truncated forms of PDZ-RhoGEF described above, lacking the PDZ domain (Δ127), the PDZ domain, and the proline-rich domain (Δ238), and the entire NH2-terminal regulatory region (Δ702). We also engineered additional deletion mutants, lacking the LH domain (ΔLH), the PH domain (ΔPH), and the DH/PH domain (ΔDH/PH), as depicted in Fig. 6A. All deletion mutants were efficiently expressed when transfected into 293T cells (see above and Fig. 6A). However, when co-expressed with an activated epitope-tagged form of Ga12 and Ga13, we found that all PDZ-RhoGEF deletion mutants lacking the LH domain were unable to associate in vivo with these G protein α subunits (Fig. 6B). These data indicate that the ability of PDZ-RhoGEF to bind heterotrimeric G proteins of the Ga12 family is strictly dependent upon the structural integrity of its LH domain.

To further study the role of the LH domain in signaling, we investigated the ability of each deletion mutant to activate SRE, a Rho-dependent event. As shown in Fig. 6C, the PH and DH domains are strictly required to induce SRE, and as described above, deletion of the PDZ and PDZ + proline-rich domains did not affect the ability of PDZ-RhoGEF to stimulate SRE. However, deletion of the entire NH2-terminal regulatory region or the LH domain enhanced the activity of PDZ-RhoGEF. Thus, these data strongly suggest that the NH2-terminal regulatory region exerts a negative regulatory activity on the catalytic DH/PH domains, and that the LH domain may be responsible for this inhibitory activity.

A DH and PH Deletion Mutant of PDZ-RhoGEF Prevents Signaling from Gaαq and Gaαqα13 to Rho-dependent Pathways—The availability of biochemically inactive PDZ-RhoGEF mutants prompted us to ask whether they can affect signaling from Gaαq and Gaαqα13 to SRE, a Rho-dependent event (27). For these experiments, we chose to use 293T cells as the transfection efficiency in these cells is greater than that in NIH 3T3 cells, thus allowing us to control the expression of each transfected DNA construct. As shown in Fig. 7, expression in 293T...

![Comparison of the amino acid sequences of PDZ-RhoGEF, p115-RhoGEF, and RGS14.](image)
cells of PDZ-RhoGEF lacking the DH/PH domains or the entire NH₂-terminal region + DH domain (Δ-956) did not affect SRE activation by RhoQL, when used as a control. In contrast, the PDZ-RhoGEF DH/PH deletion mutant specifically blocked SRE activation by Ga₁₂ and Ga₁₃. Similarly, this PDZ-RhoGEF mutant diminished the activation of SRE when mediated by lysophosphatidic acid receptors (data not shown). Thus, PDZ-RhoGEF ΔDH/PH behaves as a dominant negative mutant, probably by preventing the coupling of Ga₁₂ and Ga₁₃ to the endogenously expressed PDZ-RhoGEF or to other related GEFs.

**DISCUSSION**

Although small GTP-binding proteins of the Rho family play a critical role in a variety of cellular functions, including the organization of the actin cytoskeleton and the activity of biochemical routes regulating gene expression and cell growth,
how these GTPases are activated by cell surface receptors is still largely unknown. Thus, we investigated whether novel exchange factors for Rho-related GTPases might exist, exhibiting functional domains suggestive of a role in signal transduction. For this, we took advantage of the observation that all known GEFs for Rho proteins exhibit a DH domain, a 250-amino acid stretch of significant sequence similarity to Dbl, a transforming protein that was originally isolated from a diffuse B cell lymphoma (46). Using a consensus sequence for DH domains of Rho-exchange factors (2) to search DNA databases, we identified a yet uncharacterized molecule exhibiting a putative DH domain. Detailed analysis of its primary sequence revealed that this molecule contained additional areas of similarity with known modular domains, including a PH domain, a PDZ domain, a proline-rich region, and an area of homology to p115-RhoGEF, DRhoGEF2 and Lsc, that was not found in any other GEF described so far, and that was termed LH domain. As many of these protein regions are likely candidates to participate in signal transmission, we decided to investigate further this novel putative exchange factor.

When the DH domain of this new molecule was compared with those of other DH containing proteins, we found that it was highly related to that of two Rho-specific GEFs, p115-RhoGEF (30) and Lsc (31), and to a recently described Drosophila Rho GEF, DRhoGEF2 (33). However, PDZ-RhoGEF was more distantly related to the DH domain of exchange factors

![Fig. 6. Interaction of PDZ-RhoGEF with heterotrimeric G protein α subunits through the LH domain. A. Structure and expression of deletion mutants of PDZ-RhoGEF. Structure of the proteins encoded by the expression plasmids used in this figure are shown (left and in Fig. 2A). Δ-LH, Δ-PH, and Δ-DH/PH constructs, lacking LH, PH, and both DH and PH domains, respectively, codes for amino acid sequences in which residues 171–484, 956–1054, and 738–1054 were deleted from PDZ-RhoGEF, respectively. Lyesates from cells transfected with vector or with expression plasmid carrying an epitope-tagged PDZ-RhoGEF (PDZ-RhoGEF-AU1) and its deletion mutants were immunoprecipitated with anti-AU1 antibody and subjected to Western blot analysis with the antibody against AU1. B, 293T cells were transfected with vector or expression plasmids for Gα12QL or Gα13QL together with vector or expression vectors carrying cDNAs for wild type and deletion mutants of PDZ-RhoGEF, as indicated. Lyesates, prepared as described under “Experimental Procedures,” were immunoprecipitated with anti-AU1 antibody and subjected to Western blot (WB) analysis using specific antiserum for heterotrimeric G proteins of the Gα family. Total cellular lyses (TCL) were also subjected to Western blot analysis using the same antiserum. In A and B, bands were visualized by the enhanced chemiluminescence technique using the appropriate horseradish peroxidase-conjugated goat antiserum. C, effects of wild-type and deletion mutants of PDZ-RhoGEF on the activity of the SRE. NIH-3T3 cells were co-transfected with pSREmutL (1 μg/plate) and pCMV-β-gal (1 μg/plate) plasmid DNAs together with expression vectors carrying cDNAs for wild-type and deletion mutants of PDZ-RhoGEF, as indicated. Cells were processed as described under “Experimental Procedures.” 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 mean ± S.E. of triplicate samples from a typical experiment. Similar results were obtained in three independent experiments.
activating Rac1 and/or Cdc42 such as Tiam1 (47), Vav (48), Oat (49), and Dbl (46), and to those acting on Ras, including Sos (50) and Ras-GRF (51). Consistent with this observation, expression of epitope-tagged forms of this novel GEF did not elevate the activity of co-transfected HA-tagged forms of MAPK and JNK, but potently stimulated the transcriptional activity of SRF, as judged by the use of a reporter plasmid under the control of a mutated SRE (16). Furthermore, experiments with the use of botulinum C3 exoenzyme, which ADP-ribosylates and inactivates Rho, and an inactivation resistant form of Rho, Rho141, indicated that the enhanced expression from the SRE-driven plasmid was dependent on the availability of a functional Rho. Thus, taking together, the primary sequence similarity with Rho GEFs and these biochemical profiles strongly suggest that this novel DH-containing molecule, that was designated PDZ-RhoGEF, can stimulate in vivo Rho-specific pathways.

In addition to the DH domain, PH domain is present in all GEFs for Rho-related proteins described so far, located adjacent to the carboxyl end of the DH domain (18). PH domains are found in a wide variety of signaling molecules (52) and have been implicated in both protein-protein and protein-lipid interactions (53). Although a DH domain is necessary and sufficient for the exchange activity on Rho proteins in vitro (20), the integrity of the PH domain is required for the activity in vivo of this family of exchange factors (31) most likely by facilitating membrane translocation (31). Consistent with those observations, deletion of the DH domain, PH domain, or DH/PH domains abolished the biochemical activity of PDZ-RhoGEF. Similarly, PDZ-RhoGEF readily induced the appearance of foci of transformation when expressed in NIH 3T3 cells, and this activity required the presence of an intact DH and PH domain (data not shown), supporting a critical role for the DH and PH domains for the functional activity of PDZ-RhoGEF.

The most striking feature of this novel exchange factor was the presence of a PDZ domain, a protein-protein interaction domain originally identified as an area of homology between the product of the Drosophila dlg tumor suppressor gene and the synaptic protein PSD-95 (54), currently found in more than 60 distinct gene products (35). These domains can either bind specific recognition sequences such as the (S/T)XV motif at the carboxyl termini of certain proteins, or they can form hetero- or homodimers, suggesting that this modular protein-binding domain can participate in the formation of macromolecular complexes (35). Thus, the PDZ domain was expected to contribute to PDZ-RhoGEF function. Surprisingly, however, when this domain was deleted, we did not observe any demonstrable effect on the ability of PDZ-RhoGEF to induce Rho-dependent pathways. Similarly, deletion of the PDZ domain was shown not to affect the biological activities of the Rac1 exchange factor Tiam1 (55). Although, based on these results, we cannot rule out the possibility that the PDZ domain facilitates the interaction of PDZ-RhoGEF and other exchange factors with yet to be identified signaling molecules (see below), we therefore decided to focus our efforts on other putative functional domains. One such interesting domain is a stretch of 197 amino acids located...
upstream from the DH domain, that was termed LH domain for Lsc homology domain, and that is also found in Lsc, p115-RhoGEF, and DRhoGEF2 but not in any other GEF. This suggests that the LH domain represents a distinctive feature of this subgroup of exchange factors, which might bear functional relevance. Indeed, deletion of the LH domain enhanced the ability of PDZ-RhoGEF to stimulate SRE, to an extent comparable to that of a truncation mutant lacking the entire NH2-terminal regulatory region. Similarly, we found that NH2-terminal truncated or LH-deleted forms of PDZ-RhoGEF exhibit enhanced focus forming activity in NIH 3T3 cells when compared with the wild-type form.3 Thus, PDZ-RhoGEF, like other GEFs, appears to be negatively regulated by inhibitory sequences within the non-catalytic region (18), and this inhibitory function most likely resides in the LH domain.

Because of the possibility that the LH domain might have a regulatory function, we searched data bases for molecules displaying sequences related to the LH domain. Surprisingly, we found that the catalytic region of a recently described GTPase-activating proteins for heterotrimeric G proteins, RGS14, exhibited a limited sequence similarity to the LH domain. RGSs were initially identified as homologues of Sst2 proteins, which are negative regulators of pheromone signaling in yeast (56). This protein family, currently with 19 members, shares a 120-amino acid core, termed GH domain, which is essential for accelerating the rate of GTP hydrolysis on G proteins (56). RGS14, together with RGS12, represent novel members of this family, characterized for being substantially larger (~60 and ~140 kDa, respectively) than the majority of the other known RGSs (~25 kDa) (26). This data suggested that the LH domain might confer to PDZ-RhoGEF the ability to interact with G protein α subunits. Indeed, we found that PDZ-RhoGEF could form stable complexes in vivo specifically with members of the G12 family of G protein α subunits, Gα12 and Gα13, and that this interaction required the presence of an intact LH domain. Taken together, we can conclude that PDZ-RhoGEF can interact physically with a particular subset of Gα proteins, thereby providing a direct link between heterotrimeric G proteins and small GTP-binding proteins of the Rho family.

These findings may have important implications regarding the function of G proteins of the G12 family, Gα12 and Gα13. These ubiquitously expressed G proteins were discovered by M. Simon’s group upon amplification of mouse brain cDNA by polymerase chain reaction using degenerated oligonucleotides corresponding to regions highly conserved among G proteins (57). Gα12 and Gα13 exhibit 67% amino acid identity with each other, but only 35–44% of amino acid identity to α subunits of other classes, such as Gαi, Gαs, and Gαq (57). Furthermore, whereas members of the Gαq family of G proteins activate phosphatidylinositol-specific phospholipases, the Gαi family stimulate adenyl cyclases, and Gαs inhibits adenyl cyclases and activate certain phosphodiesterases and promote the opening of several ion channels (58–60), members of the Gα12 family of GTPases appear not to affect any of these second messenger-generating systems (60). In this regard, the finding that concertina (cta), a Drosophila gene involved in embryogenesis (41), and that Gα12 and Gα13 can behave as remarkably potent oncogenes (29, 42), provided early indications that this G protein class might be involved in growth regulation, albeit through poorly defined mechanisms. Intense investigation in many laboratories has recently generated a wealth of information on how Gα12 and Gα13 may act (see Ref. 61, for a recent review). In particular, one such study (62) demonstrated that activated Gα12 and Gα13, but not Gαi2 and Gαi3 or different combinations of β and γ subunits, mimicked the effect of activated RhoA on stress fibers and focal adhesion assembly, and we have recently provided evidence that Gα12 stimulates nuclear responses and cellular transformation through Rho (27). Furthermore, several studies have now provided evidence that members of the G12 family of G proteins link many G protein-coupled receptors, including receptors for lysophosphatidic acid, thrombin, thromboxane A2, and acetylcholine to the activation of Rho-dependent pathways and, in many cases, cell growth control (27, 63–66).

However, the nature of the molecules linking Gα12 and Gα13 to Rho remained largely unknown. In this regard, our present results suggest that PDZ-RhoGEF or other LH-containing RhoGEF might act downstream from these G protein α subunits in a biochemical route leading to Rho activation (see Fig. 8). Moreover, while our study was in the process of submission, it was reported that Gα13 could enhance the in vitro Rho-GEF activity of p115-RhoGEF, a distinct LH domain-containing exchange factor (67, 68). However, how the interaction between RhoGEFs and Gα12 and/or Gα13 leads to Rho activation in vivo is still unclear. For PDZ-RhoGEF, it is possible that binding of Gα12 proteins to its LH domain results in the translocation of PDZ-RhoGEF to the membrane where it can act on Rho. Alternatively, binding of Gα12 to the LH domain might de-repress the functional activity of PDZ-RhoGEF by preventing its negative modulatory effect. In addition, although the functional significance of the PDZ domain is still unclear, it is noticeable that some receptors known to induce Gα12 and activate Rho exhibit a COOH-terminal PDZ-binding motif, such as SVV in the case of both human and murine lysophosphatidic acid receptors, EDG-2 and VZG-1, respectively (69, 70). Thus, it is also possible that certain Gα12-coupled receptors might facilitate the recruitment of PDZ-RhoGEF by binding to its PDZ domain and, simultaneously, activating Gα12 proteins. These, as well as other possibilities, are under current investigation.

We can conclude that our present findings support the existence of a novel mechanism whereby the large family of G protein-coupled cell surface receptors can stimulate Rho-dependent pathways (Fig. 8). This pathway involves the activation of Gα12 and/or Gα13, which, in turn, will interact directly with Rho-exchange factors containing a Gα12/Gα13-binding region, such as an LH domain. This would result in the activation of Rho by a still unclear mechanism, thereby stimulating the activity of Rho-dependent pathways that, ultimately, would affect the cytoskeletal structure, nuclear gene expression, and cellular growth.

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