Mechanisms for Reversible Regulation between G_{13} and Rho Exchange Factors*

Received for publication, June 7, 2001, and in revised form, November 5, 2001
Published, JBC Papers in Press, November 6, 2001, DOI 10.1074/jbc.M105274200

Clark D. Wells‡, Mu-Ya Liu§, Mandy Jackson¶, Stephen Gutowski‡, Pamela M. Sternweis‡‡, Jeffrey D. Rothstein‡, Tohru Kozasa¶, and Paul C. Sternweis‡‡**

The heterotrimeric G proteins, G_{12} and G_{13}, mediate signaling between G protein–coupled receptors and the monomeric GTPase, RhoA. One pathway for this modulation is direct stimulation by G_{13} of p115 RhoGEF, an exchange factor for RhoA. The GTPase activity of both G_{12} and G_{13} is increased by the N terminus of p115 Rho guanine nucleotide exchange factor (GEF). This region has weak homology to the RGS box sequence of the classic regulators of G protein signaling (RGS), which act as GTPase-activating proteins (GAP) for G_{i} and G_{q}. Here, the RGS region of p115 RhoGEF is shown to be distinctly different in that sequences flanking the predicted “RGS box” region are required for both stable expression and GAP activity. Deletions in the N terminus of the protein eliminate GAP activity but retain substantial binding to G_{13} and activation of RhoA exchange activity by G_{12}. In contrast, GTRAP48, a homolog of p115 RhoGEF, bound to G_{12} but was not stimulated by the α subunit and had very poor GAP activity. Besides binding to the N-terminal RGS region, G_{13} also bound to a truncated protein consisting only of the Dbl homology (DH) and pleckstrin homology (PH) domains. However, G_{13} did not stimulate the exchange activity of this truncated protein. A chimeric protein, which contained the RGS region of GTRAP48 in place of the endogenous N terminus of p115 RhoGEF, was activated by G_{13}. These results suggest a mechanism for activation of the nucleotide exchange activity of p115 RhoGEF that involves direct and coordinate interaction of G_{13} to both its RGS and DH domains.

Heterotrimeric G proteins mediate signals from seven transmembrane receptors to a wide array of effectors, including adenyl cyclase, ion channels, phospholipases, and the exchange factor p115 RhoGEF (1). Every G protein is composed of a heterotrimer made up of α, β, and γ subunits. The four G protein families, G_{i}, G_{q}, G_{s}, and G_{12}, have been categorized by their sequence identity and the functional similarity of their α subunits (2, 3). The G_{13} family contains two members, α_{12} and α_{13} (4), which have been implicated in cellular transformation

* This work was supported by National Institutes of Health Grants GM31954 (to P. C. S.), GM07062 (to C. D. W.), and GM61454 (to T. K.) and by the Robert A. Welch Foundation (to P. C. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Pharmacology, University of Texas, Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390-9041. Tel.: 214-648-2835; Fax: 214-648-2971; E-mail: Paul.Sternweis@UTSouthwestern.edu.

© 2002 by The American Society for Biochemistry and Molecular Biology, Inc.

** To whom correspondence should be addressed: Dept. of Pharmacology, University of Texas, Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX 75390-9041, The Department of Neurology and Neuroscience, Johns Hopkins University, Baltimore, Maryland 21287, and the Department of Pharmacology and the Department of Anatomy and Cell Biology, University of Illinois, Chicago, Illinois 60612.

This paper is available on line at http://www.jbc.org

1 The abbreviations used are: GEF, guanine nucleotide exchange factor; LPA, lysophosphatidic acid; S1P, sphingosine 1-phosphate; DH, Dbl homology; PH, pleckstrin homology; GAP, GTPase-activating protein; GST, glutathione S-transferase; Rho, Ras homology; RGS, regulators of G protein signaling; GTPγS, guanosine 5′-O-(thiotriphosphate); CMV, cytomegalovirus; αα, amino acid(s).
Bi-directional Interaction of G13 and p115 RhoGEF

Go13 (26). Most interestingly, p115 RhoGEF is also an effector of Go13, which increases the activity of p115 RhoGEF as a guanine nucleotide exchange factor (GEF) for RhoA (1). Recently, GTRAP48 was found to be a GEF for RhoA that also binds Go13, but the functional implications of this interaction are not known (25).

In this study, the interaction of p115 RhoGEF and GTRAP48 with the Go13 family of heterotrimeric G proteins is more precisely defined. Deletion analysis of p115 RhoGEF provides evidence that regions outside of the apparent classic RGS box are required for accelerating the GTPase activity of Go13. A mechanism for stimulation of p115 RhoGEF by Go13 is suggested by the determination of a second binding site for Go13 in the tandem DH/PH domains of p115 RhoGEF. Finally, GTRAP48 was found to bind Go13 and act as a weak GAP on the α subunit, but its nucleotide exchange activity was not stimulated by Go13.

MATERIALS AND METHODS

Plasmids and Viruses for Expression of Protein—The cDNA encoding full-length p115 RhoGEF (24) was used for amplification of fragments of p115 RhoGEF cDNA as polymerase chain reaction (PCR) templates, amplified with an N-terminal EcoRI site and a C-terminal HindIII site for cloning into pCMV5-Myc (provided by Melanie Cobb), pGEX-KG (30), and a modified pTrc D expression vector as described previously (31). Briefly, the intervening sequence between the hexa-histidine tag (HIS) and the EcoRI restriction site of pTrc D (Invitrogen) was replaced with the amino acids Met-Gly-Ala. Fragments were transferred via EcoRI/XbaI sites from pCMV5 into pVL1902-EE (31), which contains an N-terminal EE tag (EYMPME) (24). Baculoviruses were produced through co-transfection of SF9 cells with pVL1902-EE vectors and BakPak6 that was digested with Bsa361I (CLONTECH). The N-terminal DNA fragments of p115 RhoGEF were amplified by PCR and cloned into pGEX-KG and pTrc D. The different pieces of p115 RhoGEF are named by the primers used for their amplification. All cDNA constructs were sequenced to confirm correct amplification and construction.

Expression and Purification of Proteins—All GTRAP48 and p115 RhoGEF proteins were expressed via baculovirus in cultured Spodoptera frugiperda (SF9) cells or in the transformed BL21(DE3) strain of Escherichia coli. Recombinant EE-tagged proteins were expressed in SF9 cells after infection with baculovirus. The expressed proteins were purified from lysates by affinity chromatography with anti-EE coupled Sepharose (BAbCO) as described elsewhere (32). N-terminal fragments of p115 RhoGEF and GTRAP48 were produced in E. coli as either HIS-tagged fusion proteins or as chimeras with GST (glutathione transferase). GST-tagged proteins were purified by chromatography using glutathione-Sepharose (Amersham Biosciences, Inc.) and solution B (25 mM NaH2PO4, pH 7.5, 1 mM dithiothreitol, 5 mM EDTA, 0.05%polyoxyethylene 10-laurelether, 5 μM GTP, and 50 mM NaCl). The purified GST-tagged domain were bound to 20 μl of solution containing 20 μM EE peptide (EYMPME) to remove free [35S]GTP. Samples were then rapidly gel-filtered by centrifugation at 4°C through 2 ml of Sephadex G50 resin that was previously equilibrated with buffer C (50 mM NaH2PO4, pH 8.0, 1 mM dithiothreitol, 5 mM EDTA, and 0.05%polyoxyethylene 10-laurelether) to remove free [γ-35S]GTP and [35S]GTP. Hydrolysis of GTP was initiated by adding 60 μM of the treated Go13 (about 2 pmol of loaded Go13) to buffer C containing 8 mM MgSO4, 1 mM GTP, and the proteins were being centrifuged. After incubation for the indicated times at 4°C, aliquots (50 μl) were quenched with 750 μl of 5% (w/v) Norita in 50 mM NaH2PO4. The mixtures were then centrifuged at 2000 rpm for 5 min, and 500 μl of supernatant containing [35S]GTP, was counted by liquid scintillation spectrometry.

RESULTS

Mutants of p115 RhoGEF and GTRAP48 were used to identify the domains responsible for binding to Go13, stimulation of RhoA exchange activity by Go13 and acceleration of GTPase activity of Go13 and Go13. Schematic representations of these mutants are shown in Figs. 1A and 4A. Purified proteins de-
The nomenclature in Materials and Methods allows for different purified p115 RhoGEF pieces from representative preparations (see "Materials and Methods") used for this study were separated by SDS-PAGE and stained with Coomassie Blue R-250 dye.

Derived from these constructs after expression via baculovirus infection of SF9 cells or expression in E. coli are shown in Figs. 1B and 4B.

Expression of the p115 RhoGEF rgRGS Domains—Stable expression of the N terminus of p115 RhoGEF (aa 1–248) as a fusion protein with GST has been shown previously, and aa 45–161 were predicted to form an RGS domain (26). However, subsequent deletion mutants of this N terminus identified two areas outside of the predicted RGS box that were necessary for stable expression of protein. Because p115 RhoGEF was successively deleted from the N terminus by 7 or 13 amino acids, the level of expression remained stable. Further deletion up to aa 17 or 21 resulted in fragments that did not express intact protein. This N-terminal region, which apparently interferes with protein stability, ends at or before amino acid 25, because a fragment of p115 RhoGEF encoded by aa 25–252 can be expressed at 100–200 μg/gram of E. coli (wet weight). The deletion of the first 41 amino acids resulted in a domain, p115 RhoGEF (aa 42–252), which was expressed at levels comparable to domains with intact N termini.

Disruption of the region lying C-terminal to the predicted RGS box that lies between amino acids 161 and 252 also affected expression of protein. Protein fragments starting with aa 1 and ending at aa 248 or 252 express extremely well in E. coli (3–4 mg/g of packed wet cells). However, p115 RhoGEF (aa 1–215) could only be expressed poorly at 30–40 μg/g (wet weight), and this was only possible in SF9 cells. Fragments of p115 RhoGEF with shorter C termini, which encoded either aa 25–190 or aa 42–170, showed little to no detectable expression in either bacteria or SF9 cells (data not shown).

Binding of p115 RhoGEF rgRGS Mutants to G13—The p115 rgRGS fragments consisting of aa 1–252, 25–252, and 42–252 were expressed and purified as fusion proteins with an N-terminal GST domain (Fig. 1A). All three bound preferentially to the activated form of G13 (Fig. 2A). The relative binding affinity of p115 rgRGS (aa 1–252) and (aa 42–252) for G13 was then assessed by competitive binding (Fig. 2B). HIS-tagged p115 rgRGS (aa 1–252) and (aa 42–252) proteins were added at increasing concentrations to compete with a fixed amount of the immobilized GST-tagged constructs for binding of G13. 60 pmol of HIS-tagged p115 rgRGS (aa 1–252) effectively reduced binding of 20 pmol of immobilized GST-tagged p115 rgRGS (aa 1–252) to G13. In contrast, 60 pmol of p115 rgRGS (aa 42–252) had little effect, and higher concentrations (180 and 540 pmol) were needed to substantially reduce binding of the G13 to 20 pmol of immobilized GST-tagged p115 rgRGS (aa 1–252). Similarly, HIS-tagged p115 rgRGS (aa 1–252) was a much more effective inhibitor of binding of G13 to immobilized GST-tagged p115 rgRGS (aa 42–252) than the HIS-tagged p115 rgRGS (aa 42–252). Both comparisons demonstrate that p115 rgRGS (aa 1–252) bound to G13 with 5- to 10-fold greater avidity than the truncated rgRGS of p115 RhoGEF (aa 42–252). Thus, the N-terminal residues of p115 rgRGS are not necessary for binding to G13, but do contribute significantly to the affinity of this interaction.

Definition of the Minimal p115 RhoGEF rgRGS Domain

Required for Acceleration of G13 GTPase—Several N-terminal fragments of p115 RhoGEF, which included aa 1–252, 25–252, 13–252, 25–252, 42–252, and 1–215 (described in Figs. 1A and 3A), were tested for their ability to stimulate the GTPase activity of G13. Single-turnover assays were utilized, which measure the release of [32P]P from [32P]GTP that had been pre-bound to G13. Assays were performed as described by Singer et al. (33), with the modifications outlined under "Materials and Methods." As shown previously for a GST fusion protein containing the first 246 amino acids of p115 RhoGEF (26), the p115 rgRGS (aa 1–252) stimulated the GTPase activity of G13 as well as the full-length exchange factor (Fig. 3B). However, a protein with further truncation at its C terminus, p115 rgRGS (aa 1–215), was less effective.
stimulator of the GTPase activity of Go13. Removal of additional C-terminal residues resulted in unstable proteins that had no detectable stimulation of the GTPase activity of Go13 (data not shown).

The effects of N-terminal deletion are shown in Fig. 3 (C–F). Removal of the first 5 or 12 N-terminal amino acids, p115 rgRGS (aa 6–252) or p115 rgRGS (aa 13–252), respectively, did not alter GAP activities toward Go13 (Fig. 3C). Removal of the first 17 or 21 N-terminal amino acids did not allow expression of protein domains as discussed previously. P115 rgRGS (aa 25–252) was expressed modestly as a viable domain. Although an active GAP, the potency of this construct was only about 0.1% that of the full-length rgRGS domain encoded within aa 1–252 (Fig. 3, D–F). Finally, deletion of the N terminus up to the predicted RGS box, p115 RhoGEF (aa 42–252), produced a fragment that expressed well (see above). Despite binding strongly to Go13 (about 10–20% as well as p115 rgRGS (aa 1–252), see Fig. 2B), this fragment had essentially no GAP activity toward Go13 (Fig. 3, C–E). The hint of activity observed at 10 μM (Fig. 3E) is similar to nonspecific effects of adding other control proteins and is not increased at higher concentrations of p115 rgRGS (aa 42–252).

Binding of GTRAP48 to Go13 and Go12—GTRAP48 has been shown to bind to Go13 (25), but the functional consequences and potential interaction with Go12 are unknown. The domain arrangements of GTRAP48 and p115 RhoGEF are compared in Fig. 4A, and schematic descriptions of various constructs are shown. In a chimeric protein, N48C115, the N-terminal rgRGS region of p115 RhoGEF is replaced with the homologous region from GTRAP48. Examples of purified proteins that were expressed via these constructs with EE or GST tags are shown in Fig. 4B.

The ability of GTRAP48 to bind to Go12 as well as Go13 was assessed by immunoprecipitation after transient expression in COS cells. COS cells were transfected with either myc-tagged GTRAP48 or myc-tagged p115 RhoGEF and either constitutively active Go13 (Q226L) or constitutively active Go12 (Q229L). Both p115 RhoGEF or GTRAP48 bound to Go13 in the presence of AlF4−. Although p115 RhoGEF also bound well to Go12, the interaction of this α subunit with GTRAP48 is apparently weaker and was hard to detect (Fig. 5A, see “Discussion”). The dependence of association of these GTPase-deficient forms of Go12 and Go13 on AlF4 may seem surprising. However, this reflects both the multiple states of the α subunits upon lysis of cells in GDP and slow conversion of Go-GTP to Go-GDP through slow hydrolysis or nucleotide exchange over...


FIG. 5. Binding of GTRAP48 to G13 and G12. A, myc-tagged GTRAP48 or p115 RhoGEF were co-transfected into COS cells with either constitutively active G13(Q22NL) or G12(Q219NL). After expression and lysis of cells, the exchange factors were immunoprecipitated with antibodies specific for the myc tag in the presence or absence of aluminum fluoride as described under “Materials and Methods.” Immunoprecipitates were separated by SDS-PAGE, and the relative amount of exchange factor was visualized by immunoblot (IB) analysis using an anti-myc antibody (top panel). The amount of G12 or G13 that co-immunoprecipitated with exchange factor was visualized with their respective specific polyclonal antibodies (bottom panel). B, binding of constructs encoding the rgRGS domain of GTRAP48 to G13, 70 pmol of GST-tagged GTRAP48 (aa 289–539), GTRAP48 rgRGS (aa 289–495), or p115 rgRGS (aa 1–252) were immobilized on glutathione-Sepharose and incubated with 50 pmol of purified G13 in the presence or absence of aluminum fluoride. The relative amount of G13 bound to the rgRGS constructs after washing the Sepharose beads is shown by immunoblot analysis using B860 antisera. C, the relative binding affinities of p115 RhoGEF, p115 rgRGS (aa 1–252), GTRAP48, and GTRAP48 (aa 289–539) for G13 were compared by incubating increasing concentrations of each piece with 20 pmol of immobilized GST-tagged p115 rgRGS (aa 1–252) and 10 pmol of G13. The relative amounts of G13 bound to GST p115 RhoGEF (aa 1–252) after washing were detected by immunoblot analysis using B860 antisera.

The relative affinities of GTRAP48, a fragment of GTRAP48 (aa 289–539), and p115 RhoGEF for G13 were assessed by competitive binding with immobilized GST-tagged p115 rgRGS aa (1–252) (Fig. 5C). Purified full-length p115 RhoGEF and HIS-tagged p115 rgRGS (aa 1–252) effectively attenuated binding of activated G13 to the immobilized p115 rgRGS at stoichiometries of 5- to 10-fold over the immobilized domain. In contrast, EE-tagged GTRAP48 or its HIS-tagged rgRGS domain, GTRAP48 (aa 289–539), at concentrations of 5- to 10-fold over the GST-tagged p115 rgRGS (aa 1–252) showed little competition for binding to G13. Thus, although the GTRAP48 rgRGS domain bound G13, it did so with a definitively lower affinity than the p115 rgRGS domain.

GTRAP48 is a Poor GAP for G13—The ability of GTRAP48 to stimulate the GTPase activity of G13 in a single-turnover assay is shown in Fig. 6A. Both GTRAP48 and N46C115 (a chimera derived from GTRAP48 and p115 RhoGEF; Fig. 4A) displayed low but significant stimulation of the GTPase activity of G13. However, their activities were substantially less than the stimulation obtained with an equivalent amount of p115 RhoGEF. The N-terminal fragments of GTRAP48, which contain its rgRGS domain, failed to stimulate the GTPase activity of G13 at the concentrations tested (Fig. 6B). Thus, by this in vitro measure, the rgRGS domain is only a poor GAP for G13 at best. This contrast with the robust GAP activity of p115 rgRGS.

Identification of a Second Binding Site for G13 within p115 RhoGEF—The N-terminal 248 amino acids in p115 RhoGEF are known to interact with G13 (1, 26). Interestingly, fragments of p115 RhoGEF that did not contain the rgRGS region, but did contain the DH and PH domains, also bound G13 and G12 (Fig. 7A). As expected, all pieces of p115 RhoGEF that contained the rgRGS domain, p115 wild type, p115 RhoGEF (aa 1–760), and p115 RhoGEF (aa 1–637) bound the activated form of G13 in these pull-down assays. Fragments representing the C-terminal tail (aa 760–912) and the DH domain (aa 288–637) did not bind G13. A fragment encoding the PH domain of p115 RhoGEF (aa 637–760) also did not bind G13 (data not shown). However, a fragment of p115

the extensive timeframe required for the immunoprecipitation.

To define the regions in GTRAP48 responsible for binding G13, two segments, which included sequences homologous to the rgRGS domain of p115 RhoGEF, were expressed and examined. The first segment encoded aa 1–539, which includes the PDZ-and proline-rich domains that precede the rgRGS region, but did contain the DH and PH domains, and the second segment encoded aa 539–912, which included sequences homologous to the PH domain of p115 RhoGEF (Fig. 4). Both constructs of GTRAP48 were found to be preferentially bind the activated form of G13 (Fig. 5B).
**Bi-directional Interaction of G13 and p115 RhoGEF**

The apparent turnover rate for each p115 RhoGEF construct is based on the measured number of moles of GTP·S bound to RhoA. This was determined by measuring the amount of RhoA that bound [35S]GTP·S over time and at various concentrations of exchange factor as described under “Materials and Methods.” The rates calculated 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 rate measurements were made either in the presence or absence of 200 nM Ga13, which was activated with aluminum fluoride. The last column indicates the -fold activation over basal activity affected by Ga13. The basal rates of turnover for these proteins were reported previously (31). A coefficient of determination, R², that measured the degree to which the derivatives of the apparent rates fit a linear regression model was 0.99 or greater except for the Ga13-stimulated rate of p115 RhoGEF (aa 288–760), which was 0.98.

### TABLE I

| p115 RhoGEF constructs | Basal rate | Rate in the presence of Ga13 | Activation |
|------------------------|------------|-----------------------------|------------|
| p115 RhoGEF full-length | 140        | 400                         | 2.9        |
| p115 RhoGEF Δ252–288    | 30         | 150                         | 5.0        |
| p115 RhoGEF (aa 1–760)  | 14         | 50                          | 3.6        |
| p115 RhoGEF (aa 1–637)  | 3          | 11                          | 3.7        |
| p115 RhoGEF (aa 42–912) | 70         | 180                         | 2.6        |
| p115 RhoGEF (aa 288–760)| 7          | 7                           | 1.0        |

The apparent turnover rate for each p115 RhoGEF construct is based on the measured number of moles of GTP·S bound to RhoA. This was determined by measuring the amount of RhoA that bound [35S]GTP·S over time and at various concentrations of exchange factor as described under “Materials and Methods.” The rates calculated 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 rate measurements were made either in the presence or absence of 200 nM Ga13, which was activated with aluminum fluoride. The last column indicates the -fold activation over basal activity affected by Ga13. The basal rates of turnover for these proteins were reported previously (31). A coefficient of determination, R², that measured the degree to which the derivatives of the apparent rates fit a linear regression model was 0.99 or greater except for the Ga13-stimulated rate of p115 RhoGEF (aa 288–760), which was 0.98.

GTRAP48 is also an exchange factor with specificity for RhoA (25). However, Ga13 did not stimulate RhoA exchange mediated by GTRAP48 (Fig. 8A). To test whether the rgRGS domain of GTRAP48 was capable of mediating stimulation by Ga13, it was used to replace the native rgRGS domain of p115 RhoGEF (see N48C115 in Fig. 4F for details). Interestingly, the RhoA nucleotide exchange activity of the N48C115 chimera was activated 3- to 4-fold by Ga13 (Fig. 8B). This indicates that the GTRAP48 rgRGS domain can mimic this function of the p115 rgRGS domain in the context of the rest of the p115 RhoGEF molecule.

**DISCUSSION**

The rgRGS Domain of p115 RhoGEF Is Unique from the Classic RGS Proteins—RGS proteins were originally identified in genetic screens as negative regulators of G protein signaling (35–37). The majority of RGS proteins were subsequently cloned by degenerate PCR using primers based on the RGS boxes of these founder members (15). The lack of sequence identity between the RGS and rgRGS domains would explain why the latter proteins were not identified by strategies using PCR or homology searching of data bases. The elucidation of the N terminus of p115 RhoGEF as a GAP for Ga12 and Ga13 (26) led to the suggestion of potential structural relationships with the RGS family and initiated identification of the subfamily of highly homologous rgRGS domains in PDZ RhoGEF (KIAA0380) (23), LARG (KIAA0382) (22), and GTRAP48 (25).

A region, designated the RGS box (about 110 amino acids), has been shown to be sufficient for the GAP activity (38) of several members of the RGS family, including RGS4, GAIP, and RGS10. The studies reported here demonstrate that this is not the case with the p115 rgRGS domain. Secondary structure analysis of p115 RhoGEF suggested that amino acids 45–161 would likely comprise an RGS box (26). Initial observations...
showed that aa 1–246 of p115 RhoGEF possessed GAP activity equivalent to the full-length enzyme (26). Reduction of the C-terminal end of this piece by 31 amino acids, p115 RhoGEF (aa 1–215), results in poor expression and reduced GAP activity. Further truncation of C-terminal residues results in proteins that express very poorly and have no measurable GAP activity (data not shown). Deletion of the N-terminal 25 amino acids of p115 RhoGEF reduced GAP activity over 99%. Removal of the N-terminal 41 amino acids still allowed expression of this domain but reduced binding to G_{13} by 80–90% (Fig. 2B) and completely eliminated GAP activity (Fig. 3, C–F). Thus, an RGS region of p115 RhoGEF that retains some catalytic function requires ~200 residues (aa 26–216); full function requires more.

These data indicate significant differences between this rgRGS domain and the classical RGS domains. First, the rgRGS domain requires an extended C terminus for stability and, perhaps, function. This is supported by the recent elucidation of crystallographic structures for the rgRGS domain of p115 RhoGEF (29) and the rgRGS domain of PDZ RhoGEF (39). Both of these rgRGS domains show similarity to the RGS box structure (15, 19–21) in their core regions (aa 45–161 of p115 RhoGEF) but also show clear association of these core regions with C-terminal residues that form three helices and fold back onto the core. The capability of p115 RhoGEF (aa 42–252) to bind, but not act as a GAP on G_{13}, offers a clear dissociation of these two activities. In contrast, mutational analysis of classical RGS proteins indicates that reductions in GAP activity correlate much more strongly with decreases in binding avidity between the RGS and the targeted G protein α subunit (40). Because RGS4 binds and allosterically stabilizes the transition state of the switch 1 and switch 2 domains of G_{13}, it is hypothesized that any reduction in binding energy would also reduce the degree of stabilization of the transitions state (15, 40, 41). The clear dissociation of binding and GAP activity in the p115 rgRGS indicates that it has a novel mechanism of accelerating the GTPase activity of G_{12} and G_{13}.

Comparison of the rgRGS Domains of GTRAP48 and p115 RhoGEF—GTRAP48, a recently characterized protein with an apparent rgRGS domain, was previously shown to bind G_{13} (25). Binding of GTRAP48 to G_{13} was confirmed in the current studies, but binding to G_{12} was not readily detected (Fig. 5). GTRAP48 could act as a very poor GAP for G_{12} (data not shown). Thus, GTRAP48 can interact with G_{12}, albeit weakly. The GAP activity of GTRAP48 for G_{13} was also poor and truncated proteins that contained its rgRGS region and could also bind G_{13} had little to no GAP activity (Fig. 6). Similar to GTRAP48, the rgRGS domain of PDZ RhoGEF also interacted with G_{13} and G_{12} and was a very poor GAP for G_{12}. This latter phenotype contrasts with the rgRGS region of p115 RhoGEF encoded within aa 1–252, which is as good as the full-length p115 RhoGEF at activating the GTPase activity of G_{13}.

The lack of identity between the highly negatively charged N-terminal region of p115 rgRGS (aa 26–41) and the matching region of GTRAP48 (aa 314–326) may provide one explanation for the observed low activity of GTRAP48. Removal of these amino acids eradicates the GAP activity of p115 RhoGEF. These findings then suggest that GTRAP48 is not a major GAP for the G_{12} family. Alternatively, it is possible that another factor, yet to be identified, provides the functional equivalent of the N-terminal residues (aa 26–41) in the p115 rgRGS to effect stimulation of GAP activity by GTRAP48.

The Mechanisms of p115 RhoGEF for GAP Activity on G_{13} and Mediation of Rho Exchange Activity by G_{13} Are Different—Initial experiments indicated that the N-terminal region of p115 RhoGEF, which encompasses the rgRGS domain, was sufficient for GAP activity (26). The inability of p115 RhoGEF (aa 288–760) but not of p115 RhoGEF (aa 1–760) to be stimulated by G_{13} indicated that the first N-terminal 288 amino acids of p115 RhoGEF (which contains the rgRGS domain) are important for this process. What is the role of the GAP activity in this regulation? The observation that removal of the N-terminal 41 amino acids in p115 RhoGEF eradicates GAP activity, but has no effect on the ability of activated G_{13} to stimulate exchange activity, indicates both structural and functional divergence in these activities. Thus, the stimulation of GTPase activity has no impact on the mechanism for stimulation of exchange activity and should only impact rates of inactivation.

Interactions between G_{13} and Regions Outside the rgRGS Domain of p115 RhoGEF Play a Role in Activation of Exchange Activity by G_{13}—The abrogation of regulation upon removal of its rgRGS domain suggests three mechanisms by which the exchange activity of p115 RhoGEF is stimulated by G_{13}. One mechanism would utilize the interaction of G_{13} with the rgRGS region to alleviate an autoinhibitory action of the domain on exchange activity. This is most unlikely, because truncation of this rgRGS actually caused a reduction in basal exchange activity (31) rather than an increase that would be expected from removing an autoinhibitory constraint. The more rigorous characterization reported here contrasts with an initial observation that the activity of a p115 RhoGEF fragment lacking the N terminus (aa 246–912) was greater than that of the full-length protein. One reason for this discrepancy is the use of prenylated RhoA in the current studies. Preny-...
Bi-directional Interaction of G13 and p115 RhoGEF

...lated RhoA is a much more potent substrate for p115 RhoGEF (32) than the non-prenylated GTPase used previously. P115 RhoGEF (aa 246–912) exhibits less of an increase in activity toward the prenylated form of RhoA than wild type p115 RhoGEF and, therefore, is less active than p115 RhoGEF in this context (data not shown).

A second mechanism would use binding of Gα13 to the rGGRS domain to induce an allosteric mechanism for activation by which the rGGRS region causes higher nucleotide exchange activity of the DH domain on RhoA. In a third scenario, binding of the rGGRS domain to Gα13 helps stabilize interaction of Gα13 with another part of p115 RhoGEF to promote a higher activity state of the DH domain.

The discovery that Gα13 binds to a second region of p115 RhoGEF outside of the rGGRS domain suggests that the third mechanism is most likely. Because the rGGRS domain alone provides the same GAP activity as the full-length protein (Fig. 3), this second site of interaction is clearly not needed for this function and is more likely to play a key role in the stimulation of exchange activity. The location of this second site appears to be in the area of the DH domain. A construct composed essentially of the DH and PH domains, p115 RhoGEF (aa 288–760), binds Gα13 better than to a comparable construct missing the PH domain, p115 RhoGEF (aa 288–637) (Fig. 7, A and B), or to the PH domain alone (data not shown). However, the ability of Gα13 to effectively stimulate a truncated protein that contained the rGGRS and DH domains but lacked the PH domain, p115 RhoGEF (aa 1–637), suggests that this second site of interaction is still present. The higher affinity observed when the PH domain is present may be due to stabilization of the DH domain and preservation of higher affinity for Gα13. The drastically reduced basal exchange activity of p115 RhoGEF (aa 1–637) shown previously (31) (and in Fig. 8A) and the apparent low affinity of constructs lacking the PH domain for RhoA (31) are consistent with this interpretation.

Although GTRAP48 has rGGRS and DH domains that are similar in sequence and arrangement to the corresponding domains in p115 RhoGEF, Gα13 does not stimulate the exchange activity of GTRAP48, in vitro. The physiological implication of this difference is not known. It is possible that the interaction of Gα13 with GTRAP48 could be stimulatory in vivo by mediating localization of the exchange factor or that another factor may be required to mediate a regulatory effect of Gα13 on GTRAP48. Alternatively, it is possible that G13 does not regulate the activity of GTRAP48 in the cellular milieu. Attempts to directly answer this question by overexpression of the exchange factor and Gα13 have not yet yielded definitive results for two major reasons. Expression of GTRAP48 has been highly variable, especially when co-expressed with Gα13 (e.g. Fig. 5A, compare lanes 1 and 2 with lanes 3 and 4). Furthermore, the expression of Gα13 alone gives robust activation of Rho and downstream events. The use of alternative and better controlled expression systems may eventually allow better analysis of this putative regulation.

The differential response of GTRAP48 and p115 RhoGEF to Gα13 may be explained by dissimilarities between the rGGRS or DH domains of these two exchange factors, because these domains in p115 RhoGEF are sufficient for activation of exchange activity by Gα13. To address this question, the rGGRS domain of p115 RhoGEF was replaced with the rGGRS domain of GTRAP48 to make the N48C115 chimera. The ability of this chimera to be stimulated by Gα13 just like wild type p115 RhoGEF strongly argues that the rGGRS domains of these two proteins are functionally equivalent when mediating activation of exchange activity by Gα13. Thus, differences within GTRAP48 and p115 RhoGEF that lie outside the rGGRS domain must explain their disparate responses to Gα13. The rGGRS domain of p115 RhoGEF may, therefore, function to tether and/or position Gα13 so that it can directly interact with the DH domain of p115 RhoGEF. Alternatively, interaction with the rGGRS may induce conformational changes in Gα13 that yield an ability to stimulate exchange.

Acknowledgements—We thank Jana Hadad for superb technical support, Bill Singer for comments on the manuscript, and James Chen for advice on sequence analysis. We also thank Melanie Cobb for various cDNA constructs used in this study.

REFERENCES

1. Hart, M. J., Jiang, X., Kozasa, T., Rosoe, W., Singer, W. D., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998) Science 280, 2112–2114
2. Gilman, A. G. (1987) Ann. Rev. Biochem. 56, 615–649
3. Hepler, J. R., and Gilman, A. G. (1992) Trends Biochem. Sci. 17, 383–387
4. Strahmann, M. P., and Simon, M. I. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5582–5586
5. Jiang, H., Wu, D., and Simon, M. I. (1993) FEBS Lett. 330, 319–322
6. Parks, V. (1991) Cell 64, 441–458
7. Offermanns, S., Mancino, V., Revel, J. P., and Simon, M. I. (1996) Science 275, 533–536
8. Berman, D. M., Johnson, N. L., Dhanasekaran, N., and Johnson, G. L. (1995) J. Biol. Chem. 270, 24631–24634
9. Gohla, A., Harhammer, R., and Schultz, G. (1998) J. Biol. Chem. 273, 4653–4659
10. Sah, V. P., Seasholtz, T. M., Sagi, S. A., and Brown, J. H. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 459–489
11. Nobes, C., and Hall, A. (1994) Curr. Opin. Genet. Dev. 4, 77–81
12. Berman, D. M., and Gilman, A. G. (1998) J. Biol. Chem. 273, 1269–1272
13. Watson, N., Linder, M. E., Druey, K. M., Kehrl, J. H., and Blumer, K. J. (1996) Nature 383, 172–175
14. Hands, T. W., Fields, T. A., Casey, P. J., and Peralta, E. G. (1996) Nature 383, 175–177
15. Wilkie, T. M. (2000) Annu. Rev. Biochem. 69, 795–827
16. Berman, D. M., Wilkie, T. M., and Gilman, A. G. (1996) Cell 86, 445–452
17. Hepler, J. R., Berman, D. M., Gilman, A. G., and Kozasa, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 428–432
18. Tesmer, J. S., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) Cell 88, 251–261
19. de Alba, E., De Vries, L., Farquhar, M. G., and Tjandra, N. (1999) J. Mol. Biol. 291, 927–939
20. Spink, K. E., Polakis, P., and Wies, W. I. (2000) EMBO J. 19, 2270–2279
21. Offermanns, S., Mancino, V., Revel, J. P., and Simon, M. I. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1071–1077
22. Fukuhara, S., Chikumi, H., and Gutkind, J. S. (2000) FEBS Lett. 485, 183–188
23. Fukuhara, S., Muroha, C., Zobair, M., Ishigii, T., and Gutkind, J. S. (1999) J. Biol. Chem. 274, 5868–5879
24. Hart, M. J., Sharma, S., elMasry, N., Qiu, R. G., McCabe, P., Polakis, P., and Bollag, G. (1996) J. Biol. Chem. 271, 25452–25458
25. Jackson, M., Song, W., Liu, M. Y., Jin, L., Dykes-Hoberg, M., Lin, C. I., Bowers, W. J., Federoff, H. J., Sternweis, P. C., and Rothstein, J. D. (2001) Nature 410, 89–93
26. Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998) Science 280, 2109–2111
27. Cerrone, R. A., and Zheng, Y. (1996) Curr. Opin. Cell Biol. 8, 216–222
28. Lemmon, M. A., and Ferguson, K. M. (1998) Curr. Top. Microbiol. Immunol. 228, 39–74
