MAPPING THE Gα13 BINDING INTERFACE OF THE rgRGS DOMAIN OF p115RhoGEF

RUNNING TITLE: MUTATIONAL ANALYSIS OF p115RhoGEF

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Summary

Structural requirements for function of the rgRGS domains of p115RhoGEF and homologous exchange factors differ from those of the classical RGS domains. An extensive mutagenesis analysis of the p115RhoGEF rgRGS domain was undertaken to determine its functional interface with the Gα13 subunit. Results indicate that there is global resemblance between the interaction surface of the rgRGS domain with Gα13 and the interactions of RCS4 and RGS9 with their Gα substrates. However, there are distinct differences in the distribution of functionally critical residues between these structurally similar surfaces and an additional essential requirement for a cluster of negatively charged residues at the N-terminus of rgRGS. Lack of sequence conservation within the N-terminus may also explain the lack of GAP activity in a subset of the rgRGS domains. For all mutations, loss of functional GAP activity is paralleled by decreases in binding to Gα13. The same mutations, when placed in the context of the p115RhoGEF molecule, produce deficiencies in GAP activity as observed with the rgRGS domain alone but show no attenuation of the regulation of Rho exchange activity by Gα13. This suggests that the rgRGS domain may serve a structural or allosteric role in the regulation of the nucleotide exchange activity of p115RhoGEF on Rho by Gα13.
Introduction

The intrinsic guanosine triphosphatase (GTPase) activity of the α subunits of certain heterotrimeric guanine nucleotide-binding proteins (G proteins) can be stimulated by members of the Regulators of G protein Signaling (RGS) family (1-3). The catalytic activity of these GTPase Activating Proteins (GAPs) resides in an ~120-residue α-helical domain that corresponds to a consensus “RGS-box” defined by amino acid sequence similarity. RGS-box sequences show considerable diversity and can be subdivided into distinct homologous clusters (4), but all share conserved features recognizable in the three-dimensional structures of representative family members: RGS4 (5), GAIP (6) and RGS9 (7). p115RhoGEF, a Guanine nucleotide Exchange Factor (GEF) for the monomeric G protein RhoA (8,9), is the prototype of a family that includes LARG (10), Lsc (11), PDZRhoGEF (12) and GTRAP48 (13). These proteins contain regions in their amino-terminal halves with low sequence identity to RGS domains (14). In p115RhoGEF, this region extends from residue 42 to 163. Recombinant p115RhoGEF and fusion proteins containing the first 246 (14) or 252 (15) amino terminal residues of p115RhoGEF had specific GAP activity toward the α subunits of the heterotrimeric G proteins, G12 and G13, but not toward members of the Gs, Gi, or Gq subfamilies of Gα proteins. In contrast to other RGS proteins, p115RhoGEF requires elements outside of the RGS-box to function as a GAP. A construct that extends about 75 residues beyond the C-terminus of the RGS-box is the smallest fragment of the holoprotein that could be
overexpressed as a soluble protein in *Escherichia coli* although as little as 50 additional C-terminal residues allowed expression of a functional domain in eukaryotic cells. In addition to an extended C-terminus, 25 residues that precede the RGS-box were also required for full GAP activity (15). We refer to the N-, and C-terminally extended RGS-box segment of p115RhoGEF and its homologs as the rgRGS (*RhoGEF RGS*) domain.

Two members of the p115RhoGEF family, GTRAP48 (13,15) and PDZRhoGEF2, bind to Gα13 but have little or no GAP activity; these form a distinct sequence subset with respect to both the RGS-box domain and the N-terminal segment.

Structural (5,7) and mutagenesis (16-19) studies have defined three distinct regions of the RGS domain that interact with Gα and convey GAP activity. These correspond to two surface polypeptide turns that join helical segments, together with the surface of the C-terminal α helix of the RGS domain. These RGS elements directly contact the catalytic site of Gα, principally, the Switch I and Switch II segments that undergo conformational rearrangement upon hydrolysis of GTP (20). Together, these two segments in Gα subunits contain residues that participate directly in the catalytic mechanism of GTP hydrolysis, or bind the magnesium ion cofactor (21,22). The complex formed by GDP, Mg²⁺ and AlF₄⁻, which promotes an activated state of Gα (23), also mimics the penta-coordinate transition state for GTP hydrolysis (21,22). RGS proteins have been shown to bind more strongly to the GDP•Mg²⁺•AlF₄⁻ complexes of Gα subunits than to those formed with GTP analogs (24). This, together with structural
evidence, indicates that RGS proteins accelerate GTP hydrolysis by stabilizing a
transition state-like conformation of Gα or destabilize the GTP-bound ground state (25).

The crystal structures of the amino-terminally truncated rgRGS domain of
p115RhoGEF (26) and that of its homolog, PDZRhoGEF (27), were recently determined.
These domains were comprised of eleven α helices; the N-terminal seven helices form a
bilobal fold similar to that in classic RGS domains. The remaining four helices pack
against the latter to generate a single globular domain. The core of the rgRGS domain
from p115RhoGEF, which encompasses two of the three segments involved in Gα
binding, shows high structural similarity to the corresponding segments in RGS
proteins of known structure. Accordingly, mutation of two p115RhoGEF residues that
correspond, in RGS4, to side chains that interact with Switch I and Switch II, were
found to diminish GAP activity (26). These observations led to the inference that the
general features of the protein-protein interface observed in the published structures of
RGS:Gα complexes are likely be preserved in the interaction of rgRGS with Gα13.
Although the N-terminus of p115RhoGEF is not present in the crystal structure, it is
required for GAP activity (15), and structural modeling of the rgRGS:Gα13 complex
suggests that amino terminal residues of the rgRGS domain might interact with the
helical domain and switch regions of Gα13 (26).

Here, we describe an extensive mutagenic analysis performed to define the
residues in the rgRGS domain that interact with Gα13 and convey GAP activity. These
studies focused on both the globular helical domain of the rgRGS and the N-terminal residues that precede the RGS-box. Results indicate that there is global resemblance between the interaction surface of the rgRGS domain with Ga13 and the interactions of RGS4 and RCS9 for their Ga substrates. However, there are distinct differences in the distribution of functionally critical residues between these structurally similar surfaces and an additional essential requirement for a cluster of negatively charged residues at the N-terminus of rgRGS. Placement of the same mutations in the context of the p115RhoGEF molecule produces the same deficiencies in GAP activity as observed with the rgRGS domain alone but show no attenuation of the regulation of Rho exchange activity by Ga13.
Materials and Methods

Expression Plasmids—Complementary DNA oligomers encoding single or double amino acid changes from the wild type p115RhoGEF gene were used to generate point mutants by the polymerase chain reaction (PCR). The mutagenesis template consisted of amino acids 1-252 of human p115RhoGEF (rgRGS domain) subcloned into the pGEX-KG vector. The sequences of primers used are available upon request. The QuickChange Kit™ from Stratagene was used in a four-step procedure to generate mutant cDNA. Plasmids encoding the desired mutations were selected and verified by sequencing. Selected mutated rgRGS DNAs were also subcloned into the full length p115RhoGEF gene in the pFastbac1 vector (Gibco), which has been modified to provide an N-terminal EE tag (EYMPME) (8) for production of baculovirus and subsequent expression in Spodoptera frugiperda (Sf9) cells.

Expression and Purification of Proteins—All p115RhoGEF proteins were expressed in transformed BL21 (DE3) or DH5α strains of Eschericia coli or in cultured Sf9 cells infected with recombinant baculoviruses. The rgRGS domains were produced in E. coli as fusion proteins with glutathione S-transferase (GST). Cells were grown in LB medium at 37°C to OD600 ~0.6 and induced for 3 hr at 30°C with 0.2 mM isopropyl-1-thiogalactopyranoside (IPTG) for expression of mutant proteins. Bacteria were then pelleted by centrifugation and lysed for 30 minutes in solution A (25 mM Na⁺·N-(2-hydroxyethyl) piperazine-N’2-ethanesulphonic acid (NaHepes ), pH 7.5, 1 mM ethylenediamine tetracetic acid (EDTA), 1 mM dithiothreitol (DTT), 50 mM NaCl, and protease inhibitors (2.5 µg/ml leupeptin, 1 µg/ml pepstatin A, 21 µg/ml
phenylmethylsulfonyl fluoride, 21 µg/ml N\textsuperscript{-}p-tosyl-L-lysine chloromethyl ketone, 21 µg/ml tosylphenylalanyl ketone, and 21 µg/ml N\textsuperscript{-}p-tosyl-L-arginine methyl ester) containing 2 mg/ml lysozyme, 1 mg/ml DNase I, and 5 mM MgCl\textsubscript{2}. GST-tagged rgRGS proteins were purified by chromatography through Glutathione Sepharose-4B (Amersham Pharmacia Biotech) in solution A and elution with solution A containing 15 mM reduced glutathione.

Full length p115RhoGEFs containing mutated rgRGS domains were expressed in Sf9 cells during 48 hours of infection with recombinant baculoviruses. The expressed proteins were purified from lysates by chromatography with immobilized antibodies to the EE tag (BabCO) as described elsewhere (8).

G\textalpha\textsubscript{13} was expressed in Sf9 cells and purified as described previously (28). RhoA was expressed in Sf9 cells with an N-terminal hexahistidine (6H) tag and purified from lysates by isolation with Ni\textsuperscript{2+}-nitriloacetic acid resin (Qiagen) in 25 mM NaHepes, pH 7.5, 2.5 mM β-mercaptoethanol, 50 mM NaCl, and protease inhibitors. Elution was facilitated by a step gradient of increasing imidazole concentration, with 6H-RhoA being released at approximately 75 mM.

\textit{GAP Assays for G\alpha13 -- G\alpha13} (2 µM) was loaded with 5 µM [\gamma\textsuperscript{32}P]GTP (50-100 cpm/fmol) for 15 minutes at 30 °C in solution B (20 mM NaHepes, pH 8.0, 5 mM EDTA, 1 mM DTT, and 0.05 % polyoxyethylene 10 lauryl ether (Lubrol)). Free [\gamma\textsuperscript{32}P]GTP and \textsuperscript{32}P\textsubscript{i} were removed rapidly by centrifugation of the samples at 4 °C through Sephadex G-50 that had been equilibrated with solution B. Hydrolysis of GTP was initiated by adding G\alpha13 loaded with [\gamma\textsuperscript{32}P]GTP (final concentration of 1-5 nM) to solution B
containing 10 mM MgSO₄, 1 mM GTP and 1-100 nM p115RhoGEF rgRGS domain (wild type or mutant) in a 50 µl reaction volume. After incubation for the indicated times at 4 °C, reactions were quenched with 750 µl of 5 % (w/v) NoritA in 50 mM NaH₂PO₄. The mixtures were then centrifuged at 3000 rpm for 5 minutes, and 400 µl of supernatant containing ³²Pᵢ was counted by liquid scintillation spectrometry.

G Protein Binding Assays -- The purified GST-tagged rgRGS domains were bound to 20 µl of Glutathione Sepharose 4B (packed beads) by gentle mixing in 200 µl of solution C (20 mM NaHepes, pH 8.0, 1 mM EDTA, 1 mM DTT, and 100 mM NaCl) for 30 min at 4 °C. During that time, Gα₁₃ was diluted to 2 µM in solution D (20 mM NaHepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 0.1 mM GDP, and 0.05 % Lubrol) and then gel-filtered rapidly by centrifugation through Sephadex G-50 which had been equilibrated with the same solution. This removes residual activating ligands in the preparation. Samples of Glutathione Sepharose 4B containing bound GST-tagged rgRGS proteins were then pelleted in a microfuge for 30 seconds and washed with 200 µl of solution D to remove unbound protein. Binding reactions (200 µl) were prepared by mixing the immobilized rgRGS proteins and gel-filtered Gα₁₃ in solution D either with or without AMF (30 µM AlCl₃, 5 mM MgCl₂, and 5 mM NaF). Samples were mixed gently for 30 minutes at 4 °C and unbound Gα₁₃ was then removed by two 200 µl washes of solution D either with or without AMF. The washed beads were finally resuspended with 40 µl of solution D and then boiled in SDS sample buffer.
Results

*Mutations of the rgRGS helical domain that affect GAP activity towards Gα13* --

Structural modeling of the rgRGS:Gα13 complex predicted that residues of loops L3, L5, helix α8 and loop L11 interact with the switch regions of Gα13 (Fig. 1A) (26). To test this hypothesis, we mutated residues located within this predicted interaction surface of the p115RhoGEF rgRGS domain and measured the GAP activity of the mutant domains towards Gα13. Unless stated otherwise, “p115rgRGS” refers to the protein fragment encompassing residues 1-252 of p115RhoGEF. Mutations are identified by the one letter code for the residue in wild type p115rgRGS, followed by the position of the residue in the amino acid sequence and the one letter code for the residue to which it was mutated (e.g., E71A has glutamic acid in position 71 mutated to an alanine). Residues chosen for mutagenesis (Fig. 1B) fall into three categories. The first group includes residues predicted to form direct and specific contacts with the switch regions of Gα13, according to structural modeling studies of the rgRGS:Gα13 complex (Glu 71, Arg 111, Pro 113, Pro 115, Pro 116, Glu 155, and Lys 214). The second group of residues in the predicted interface region includes those that are charged, have solvent accessible surface areas greater than 25 Å², and are conserved in the rgRGS domains but not in the classical RGS proteins, (Gln 69, Asp 156, Lys 160, Arg 161, Glu 212 and Glu 213). A third group of residues includes surface residues that are predicted to project towards, but not necessarily contact, switch regions of Gα13 (Phe 70, Arg 152 and Ser 159). In most instances, residues were mutated to alanine or residues with opposite charge. Three of the mutations, F70A, P113K and R152E, were also designed to reflect the differences in
sequence between p115RhoGEF and two rgRGS-containing proteins with little or no GAP activity, GTRAP48 and PDZRhoGEF. None of the residues chosen for mutagenesis are involved in extensive packing interactions. Hence, alterations of these residues are therefore not expected to disrupt the tertiary structure of p115rgRGS.

**Insert Figure 1**

Initial experiments were conducted with constructs encompassing only the p115rgRGS domain (residues 1-252). A total of 21 mutated p115rgRGS domains were expressed in *E. coli*. All of the mutants were expressed at a high level (> 1 mg/L) and as soluble proteins (data not shown) as would be expected for properly folded protein domains. The GAP activities of the recombinant p115rgRGS mutants were measured by the single turnover GTPase assay for Gα13 (see “Materials and Methods”). Examples of these assays, which assessed the effect of 10 nM mutant p115rgRGS protein on the time-dependence at 4 °C of hydrolysis of GTP bound to Gα13, are shown in Figs. 2A,B. At this concentration and conditions, the wild type rgRGS domain stimulated the GTPase activity of Gα13 by approximately 5 - 10 fold and caused almost complete hydrolysis of the bound GTP by 2 minutes. The total amount of GTP hydrolyzed differed from assay to assay, due largely to variations in GTP loading caused by weak nucleotide affinity and the high intrinsic GTPase rate of Gα13 (29); thus, the final concentration of [γ-32P]GTP-loaded Gα13 used in the assays ranged from 1 nM to 10 nM.

**Insert Figure 2**

A summary of the results with the 21 mutant domains tested under the conditions described above is shown in Fig. 2C. Several mutations, including R111A,
E155K, E212A, E213A, K214A and K214E, had no effect on GAP activity. In contrast, three mutations, Q69A, F70A and D156A, appear to largely abolish GAP activity while others mutations had more modest effects (apparent decreases in GAP activity of 20 – 60 % of the wild type domain). GAP activities of mutants designed to target specific Gα13 interaction sites are consistent with the involvement of the structurally conserved L3 and L5 loops, and also the less well-conserved helix, α8. However, the lack of effects by mutations in L11 from the C-terminal helical region does not support a role for this structural element in the GAP activity of the rgRGS domain.

The L3 loop of the rgRGS domain is a likely site for interaction with Gα13, yet its conformation differs from that of RGS4. The group 1 residue in this loop, Glu 71, is proposed to engage in electrostatic and van der Waals interactions with Lys 204 of Gα13 and is conserved among rgRGS proteins (Fig. 1B). Accordingly, mutants in which Glu 71 was substituted with either alanine or lysine have 50 % of the GAP activity towards Gα13 compared to the wild type rgRGS domain (Fig. 2C). Non-conservative mutations of the group 2 and group 3 residues in L3, Q69A and F70A, respectively, caused large reductions in GAP activity. Both residues are exposed to solvent, and are in close proximity to Switch I of Gα13 in the model of the rgRGS: Gα13 complex, although their interaction partners cannot be predicted. Gln 69 is absolutely conserved among known rgRGS proteins (Fig. 1B) whereas Phe 70 is conserved only in p115RhoGEF, Lsc and LARG, but replaced by alanine in PDZRhoGEF and GTRAP48. The potential interactive role of this residue is supported by the retention of about 50 % of GAP activity by the conservative mutation of Phe 70 to tyrosine.
Structural and mutagenic studies of RGS proteins indicate that the residue corresponding to Asn 128 in RGS4 is important for GAP activity. A proline residue occupies the analagous position (residue 113 in p115RhoGEF) in the rgRGS domains with strong GAP activity, but is replaced by lysine in GTRAP48 (Fig. 1B). Mutation of Pro 113 in L5 to either an alanine or a lysine reduces GAP activity (Fig. 2C), but the effect is not as severe as that caused by mutations in the L3 or α8 regions. Other mutations of group 1 residues in L5, including R111A and P115G/P116G, had little effect on GAP activity (Fig. 2C).

Residues outside the α3-L5 core region of the rgRGS domain align poorly in tertiary structure with RGS4. Therefore, although α8 is a probable interaction partner for Switch I of Gα13, only one residue in this helix, Glu 155, could be included in group 1. Mutation of this residue had little effect on GAP activity. In contrast, mutation of one of the two neighboring group 2 residues, Arg 152, reduced GAP activity by 50 %. Arg 152 is replaced by glutamate in PDZRhoGEF and GTRAP48, two rgRGS proteins with very weak GAP activity towards Gα13. Substitution of Arg 152 with glutamate in p115rgRGS resulted in only a modest reduction in activity similar to replacement with alanine. Three charged group 3 residues were selected for mutagenesis in α8. Of these, substitution of Asp 156, a residue in α8 that is conserved among rgRGS proteins (Fig. 1B), severely reduced GAP activity. However, structure-based modeling reveals no residues in Gα13 poised to interact directly with Asp 156 in the rgRGS domain. Mutation of other charged residues in α8, Glu 155, Ser 159 and Arg 161, had little effect.
To elucidate the extent to which certain mutations affect GAP activity of the
rgRGS domain, we measured the quantity of GTP hydrolyzed by Gα13 within a two
minute period as a function of rgRGS concentration (Fig. 2D). At sufficiently high
concentration, all of the mutated rgRGS domains were capable of stimulating Gα13 to
the extent observed for the wild type domain. Thus, the D156A, Q69A and F70A
mutations do not substantially alter the efficacy of rgRGS as a GAP for Gα13, but
strongly reduce the potency of the domain for this function. Even the most debilitated
mutant, p115rgRGS (D156A), exhibited GAP activity that was comparable to that of the
wild type protein at concentrations that were 100-1000 fold greater than Gα13 in the
assay.

Insert Figure 3

A map of the Contact Surface of the rgRGS Core-Domain for interaction with Gα13

In Fig. 3A, the solvent -accessible surface of rgRGS is color-coded according to the
relative loss of GAP activity that results from mutation of the underlying residues. The
relative GAP activity of a mutant p115rgRGS domain is quantified as the apparent
initial velocity of the GTPase reaction catalyzed by Gα13 in the presence of the mutant
normalized by the rate in the presence of wild type p115rgRGS (see Figs. 2A,B for
examples). The mutagenesis data identify a functionally critical surface on the rgRGS
domain that is centered on three key residues from the RGS-box: Gln 69 and Phe 70
from L3, and Asp 156 from α8. The functional map of rgRGS is grossly similar to that of
RGS4 as charted by Srinivasan and colleagues (16) (Fig. 3B), but differs in the extent to
which mutations at corresponding positions affect GAP activity. For example, mutation
of Asn 128 in the L5 loop of RGS4 severely impairs GAP, while the corresponding P113A mutation is only modestly debilitating in rgRGS. Conversely, the Q69A and F70A substitutions generate severe effects in rgRGS, while mutation of the structurally equivalent residues in RGS4 only reduce GAP activity to ~30% of wild type. On the other hand, Asp156 in rgRGS and Arg167 in RGS4 occupy equivalent positions in α8, and both are critical for GAP activity of the respective domains.

_Mutations of Residues N-terminal to the RGS-box in the rgRGS Domain_ -- Residues 15-41 of p115rgRGS are required for GAP activity towards Gα13 (15). This region contains a hydrophobic sequence followed by a sixteen-residue segment containing nine acidic residues (Figs. 1B, 8A). Within this region, sequence conservation between p115RhoGEF and GTRAP48, which possesses only weak GAP activity, is low, and fewer negatively charged side chains are present in GTRAP48. To define the contribution of residues within the acidic sequences to the GAP activity of rgRGS, a series of single site mutations were generated within the segment extending from residue 27 to residue 45. Also included for mutagenesis were two amino acids at the N-terminal border of the functional rgRGS domain, Ser14 and Arg15. Within this set, negatively charged glutamic acid residues were mutated to lysine; the remaining amino acids were mutated to alanine. All of the mutant p115rgRGS domains could be expressed as soluble proteins in _E. coli_ and were assayed for GAP activities towards Gα13 as described above. Residues 27 to 31 were identified as crucial to the GAP activity of the rgRGS domain. Mutation of Glu 27 or Glu 29 to lysine severely impaired GAP activity (Figs. 4A,B) as did the mutation of Asp 28 or Phe 31 to alanine. Mutations
of the charged residues immediately flanking this segment reduced GAP activity to ~50% that of wild type, whereas mutations of residues 40-45 had no effect. Substitution of Ser 14 and Arg 15 did not reduce GAP activity appreciably. The concentration-dependence of GAP activity was also measured for p115rgRGS bearing single mutations in residues 27-31 (Fig. 4D). In contrast to mutations within the RGS-box region, mutation of these residues appeared to severely reduce efficacy as well as potency.

Insert Figure 4.

Mutations of residues required for rgRGS GAP activity also reduce affinity for Gα13 --

Recombinant p115rgRGS proteins, which possess single mutations from the N-terminus (E27K, D28A, E29K, F31A, E32K, or E34K) of the rgRGS domain or from the RGS-box region (Q69A, F70A, F70Y, E71K, or D156A), or the double mutation (E71K-P113K), were tested for their ability to bind Gα13 in a pull-down assay. Immobilized p115rgRGS proteins were incubated with Gα13 in the presence of GDP and Mg2+ or GDP with Mg2+ and AlF4- (Fig. 5). Both wild type and mutant p115rgRGS domains bound only poorly or not at all to Gα13•GDP in the absence of Mg2+ and AlF4-. Whereas the wild type rgRGS domain bound efficiently to the activated α subunit (Gα13•GDP•Mg2+•AlF4-), the E27K, D28A, E29K, F31A, and D156A mutant proteins failed to bind appreciably. In the GAP assays described in the previous sections, these mutations severely reduced GAP activity of the p115rgRGS domain. Two mutations in the RGS domain, Q69A and F70A,
that reduced the efficacy, but not the potency of p115rgRGS in the GAP assay, bound to 
G\alpha 13\cdot GDP\cdot Mg^{2+}\cdot AlF_{4}^{-}, albeit to a lesser extent than wild type p115rgRGS. Other 
mutants tested in the pull down assay, which showed only modest reduction in the 
GAP activity, retain their ability to bind G\alpha 13\cdot GDP\cdot Mg^{2+}\cdot AlF_{4}^{-}. Mutations that severely 
impair the GAP activity of rgRGS also strongly diminish its ability to bind to activated 
G\alpha 13; in no case do we observe an inactive rgRGS mutant that retains its full ability to 
bind G\alpha 13.

Insert Figures 5 and 6.

Activities of p115RhoGEF containing point mutations in the rgRGS domain -- Several 
of the single residue mutations characterized for the rgRGS domain were inserted into 
the holo-p115RhoGEF protein to determine their effect in this context. As shown in Fig. 
6A, these mutations are as debilitating to the GAP activity of the holo-protein as they 
are to that of the rgRGS domain alone. Thus, mutations F31A and E27K caused almost 
total loss of GAP activity while three other mutations (D156A, D28A, and E29K) 
showed impaired potency but substantial efficacy at higher concentrations. As with the 
rgRGS domain, these mutations also reduced the affinity of the holo-protein for 
activated G\alpha 13 (Fig. 6B). This indicates that the rgRGS domain confers the dominant 
elements required for binding of p115RhoGEF to G\alpha 13.

In contrast to the impairment of binding and GAP activities, the point mutations 
described above did not affect the ability of activated G\alpha 13 to stimulate the Rho
exchange activity of p115RhoGEF (see Fig. 7 for examples). Among the mutants tested, stimulation of exchange activity in either the wild type or mutant proteins ranged from 3-5 fold with an EC50 for Gα13 between 5-15 nM. There is no apparent decline in potency that correlates with the observed effects on activities of the rgRGS domain. Thus, while the rgRGS domain is required for regulation of Rho exchange by Gα13 (15), binding of the domain to the α subunit does not appear to be coupled to this effect.

*Insert Figure 7.*
Discussion

The rgRGS domains form a unique and distinct subgroup of the RGS family. Although the rgRGS domains are, in three-dimensional structure, apparent homologs of the RGS domains, their sequences bear little similarity to the RGS consensus sequence (3). The mutagenesis experiments that we have conducted are consistent with the hypothesis that the Gα₁₃ interaction surface of the RGS-box region in p115RhoGEF is roughly similar to that observed in the structures of RGS4 and RGS9 bound to their Gα substrates (5,7). However, the set of rgRGS residues that emerges from the analysis as “hot spots” for GAP activity are not all cognates of the functionally critical residues in RGS4 (16).

The segment extending from L3 to L5 is the most highly conserved structural feature common to the rgRGS and RGS folds. Less well conserved is the segment corresponding to α7-α8 in RGS4, which folds as a single helix, α8 in rgRGS. Residues from both segments are important for GAP activity in both the RGS and rgRGS domains. A triad of residues, Gln 69, Phe 70, and Asp 156, appears to form a functional core in rgRGS. Their counterparts in RGS4 are Glu 83, Tyr 84 and Arg 167, respectively. In the complex with Gαι1, Arg 167 is involved in a weak or indirect electrostatic interaction with the main chain of Thr 182 in Switch I, near the center of the RGS4: Gαι1 contact region. Glu 83 plays a supporting role through formation of an ion pair with Arg 167, but does not directly contact the G protein. Tyrosine 84 forms a stacking interaction with His 213 at the C-terminus of the Switch II helix in Gαι1. As is true for its counterpart, Asp 156 in p115rgRGS, mutation of Arg 167 causes a severe loss of GAP
activity (17), while the effects of mutations at positions 83 and 84 are less damaging (16). However, if we create a model of the rgRGS:Gα13 complex by superposing both components onto the corresponding structures in RGS4:Gα11 (26), none of the core triad residues directly contact Gα13. We therefore suppose that the rgRGS:Gα13 complex differs in significant molecular detail from that of RGS4:Gα11.

Mutation of Asn 128 in the L5 loop of RGS4 (16), can result in a modest reduction in GAP activity, if replaced by serine (the cognate residue in GAIP), but severe losses (>99.9 % reductions), if replaced by bulkier residues (18). Yet, mutations of the structurally equivalent residue in p115RhoGEF, Pro 113, to either alanine or lysine, decreases GAP activity only 50 % at a 10:1 molar ratio of rgRGS to Gα13. Pro 113, and possibly the L5 loop, therefore appear to be less important to the function of rgRGS than equivalent regions of RGS proteins.

The N-terminal 41 residues of p115RhoGEF include a hydrophobic and proline-rich sequence (residues 11-26) followed by a fifteen-residue segment (aa 27-41) containing nine acidic residues (Fig. 8A). Although the first 12 residues of p115RhoGEF were shown to be dispensable for GAP activity, deletion of the first 41 residues abolished this function (15). Here, we show that the electronegative cluster encompassed by residues 27-30 is crucial to GAP function. Indeed, substitution of any of the first three acidic residues in the cluster caused a greater than 99 % loss of GAP potency and efficacy. Mutation of the aromatic residue, Phe 31, which is adjacent to the acidic sequence, is equally deleterious. The only mutation within the RGS domain of rgRGS that causes an equivalent degree of impairment is that of Asp 156 in α8, which,
of the residues mutated, has the least solvent-accessible (~25 Å²) surface area in the structure of p115rgRGS. Thus, residues within the RGS-box, and a small cluster in the preceding N-terminal are both necessary for the GAP activity of rgRGS.

*Insert Figure 8.*

In the absence of structural information, we speculate that residues N-terminal to the RGS-box might function in GAP activity through an electrostatic binding mechanism. A model of the rgRGS:Gα13 complex based on that of previously determined RGS:Gα complexes shows a cluster of positively charged residues contributed by helix α8 of the rgRGS domain and the switch regions and central helix (residues 79-105) of the helical domain of Gα13. The latter form an electropositive trough that could provide a binding site for the negatively charged residues that precede the amino terminus of the rgRGS domain (Figs. 8B,C). The electropositive character of helical domain of Gα13 appears to be a conserved feature of the G12 class of Gα subunits. In Gai, Gao, Gaq and Gaz, which are not substrates for p115RhoGEF, many of the positively charged residues in the helical domain are replaced by neutral or acidic residues. The most critical residues within the RGS domain of rgRGS: Gln 69, Phe 70 and Asp 156, might be supposed to form, with the critical EDEDF segment, a functional core of the rgRGS:Gα13 interface. Phenylalanine 31, which flanks this highly charged segment, might be accommodated in a hydrophobic pocket, possibly within the helical domain of Gα13.

The rgRGS subfamily represented by GTRAP48 and PDZRhoGEF have little or no GAP activity towards Gα13. The absence of GAP activity for these two rgRGS
proteins may stem from specific sequence differences in either the RGS-box or the preceding N-terminal sequence. The overall sequence similarity between p115RhoGEF and GTRAP48 is high (57 %) in the RGS-box region (Fig. 1B) and many of the substitutions are conservative. Three mutations, F70A, P113K and R152E, were designed to reflect non-conserved differences in sequence between p115RhoGEF and GTRAP48 in regions hypothesized to form contact sites with Gα13. Of these, F70A is sufficient to severely reduce catalytic efficiency and binding affinity of the rgRGS domain towards Gα13. In contrast, differences between the N-terminal sequences of p115RhoGEF and GTRAP48 are so great that it is difficult to align them. Although both active and inactive RhoGEFs possess a sequence consisting of three or four acidic residues preceding a phenylalanine (residues 27-31 in p115RhoGEF) or tyrosine, the position of this sequence relative to the N-terminus differ. Therefore, spatial mismatch of the N-terminal acidic cluster with respect to its potential binding site on Gα13, might explain the reduced GAP activity of GTRAP48.

All of the mutations that diminish the GAP activity of p115rgRGS also reduce its affinity for Gα13. A quantitative relationship between affinity and activity cannot be deduced from the data presented here, as is the case for RGS4 (18), however, no mutants capable of binding Gα13 are devoid of GAP activity. It therefore appears that the GAP activity of p115rgRGS arises from its ability to bind and stabilize a conformational state of Gα13 that is conducive to transition state formation. The mechanism by which rgRGS acts in this role may be in part elucidated by structural studies now in progress. The requirement for specific N-terminal residues outside of the
conserved RGS-box indicate that the rgRGS domains have developed alternate mechanisms to stabilize such a state in the G12 subfamily of heterotrimeric G proteins.

The rgRGS domain contributes to the intrinsic nucleotide exchange activity of p115RhoGEF as demonstrated by the 60 % reduction of this activity when the domain is deleted (30). However the most notable effect that results from this truncation is a total loss of the ability of p115RhoGEF to be stimulated by Gα13 (15). One hypothesis is that it is the binding of the rgRGS to Gα13 that facilitates this regulation. This is consistent with the observation that the regulation of exchange activity by Gα13 was retained when the rgRGS region of GTRAP48 was substituted for the endogenous rgRGS domain of p115RhoGEF (15). Yet, as we show here, mutations that compromise the ability of the rgRGS domain to bind to Gα13 do not affect the susceptibility of p115RhoGEF to activation by Gα13. This finding is consistent with a model in which the rgRGS domain plays a structural or allosteric role, for example, by conferring stability upon conformational states of other segments of p115RhoGEF that are required for Gα13-mediated stimulation of GEF activity. The reduction of inherent GEF activity upon deletion of the rgRGS domain (30) is an evidence for direct intramolecular coupling between the rgRGS and DH/PH domains. This raises the intriguing possibility that the ability of the rgRGS domain to affect the efficacy of Gα13 might itself be subject to regulatory mechanisms such as covalent modification, which have yet to be discovered.

The data presented here also suggest that stimulation of p115RhoGEF by Gα13 results from interaction of the α subunit at a surface on p115RhoGEF different from the
rgRGS domain. Previous binding studies of Ga13 to p115RhoGEF detected a second site for interaction between residues 288 and 760, which includes the DH and PH domains (15). It is most likely the surface of Ga13 that binds the rgRGS domain and that which engages the second site are distinct. This could resemble the interaction demonstrated by Slep, et al. (7), that Ga1 binds the γ subunit of cyclic GMP phosphodiesterase and RGS9 simultaneously at separate, yet structurally interacting interfaces (7). The mechanisms by which Ga13 interacts with regions beyond the rgRGS domain of p115RhoGEF, and exploitation the coupling between this and other domains of the molecule, remain to be explored.
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Footnotes

1 The Abbreviations used are: GTPase, guanosine triphosphatase; RGS, regulator of G protein signaling; GEF, guanine nucleotide exchange factor; Rho, Ras homology; GAP, GTPase activating protein; GST, glutathione S-transferase; DH, Dbl homology; PH, Plextrin homology

2 T. Kozasa, personal communication
Figure Legends

FIG. 1. Tertiary and primary structure of p115rgRGS. A, the tertiary structure of p115rgRGS (26) is depicted using the coloring scheme published for RGS4 (5); the amino-terminal 42 residues and carboxy-terminal 19 residues of the rgRGS domain are not present in the structure. Helices that comprise structural elements of the RGS-box are colored orange, green and blue. L3, L5 and helix α8 in RGS4 and RGS9 form contacts with Gαi1 and Gαt, respectively (5,7) (see text). The C-terminal helical bundle (colored red) is unique to rgRGS domains. B, the amino acid sequences of rgRGS domains were aligned with two members of the RGS family for which crystal structures have been determined. Sequence alignments performed using Clustal W (31) were modified on the basis of structural superposition (26). Colored bars set above the sequences represent helices with color codes that match the ribbon diagram shown in panel A. Gray bars represent helices in the structure of RGS4 (5). Residues in RGS4 or RGS9 (7) that are involved in contacts (closer than 4.0Å) with Gαi or Gαt, respectively, are colored in red. Residues in rgRGS proteins that are conserved and have solvent accessible surface areas greater than 25 Å² in the structure of p115rgRGS are colored in purple, whereas those conserved but have solvent accessible surface areas less than 20 Å² are colored in blue. Residues marked with asterisks were targeted for site-directed mutagenesis.

FIG. 2. Effect of mutations within the RGS-box of p115rgRGS on GAP activity. A, Gα13 was loaded with [γ-32P]GTP (final concentration of 1-5 nM) and hydrolysis of the bound GTP was initiated by mixing with buffer containing 10 mM MgSO₄ and 1
mM GTP (basal, closed circles). Samples were incubated for the indicated times at 4 °C, and then quenched as described under “Materials and Methods”. $^{32}\text{P}_1$ evolved from the reactions was counted by liquid scintillation spectrometry. As indicated, 10 nM p115rgRGS domains were included in the reaction: wild type p115rgRGS (closed diamonds), D156A mutant (open circles), K160A mutant (closed triangles), R161A mutant (open triangles), E212A mutant (closed squares) or E213A mutant (open squares). B, the time course of GTP hydrolysis, as described in panel A, is shown for basal (closed circles), wild type p115rgRGS (open diamonds), Q69A mutant (open circles), F70A mutant (closed triangles), F70Y mutant (open triangles), R152A mutant (closed squares), R152E mutant (open squares) or S159A mutant (closed diamonds). C, the apparent initial rate of GTP hydrolysis catalyzed by G$\alpha$13 in the presence of each p115rgRGS mutant (10 nM) is represented as a percentage of the initial rate of GTP hydrolysis by G$\alpha$13 in the presence of 10 nM wild type p115rgRGS. D, the quantity of $^{32}\text{P}_1$ released in the assay as described in panel A after two minutes of incubation was determined in the presence of the indicated concentration of mutant or wild type p115rgRGS: wild type p115rgRGS (closed circles), Q69A mutant (open circles), F70A mutant (closed triangles), E71K mutant (open triangles), D156A mutant (closed squares) or E71K-P113K mutant (open squares).

FIG. 3. Relative GAP activity of p115rgRGS and RGS4 mutants mapped on the G$\alpha$ binding surface. A, the solvent accessible surface of p115rgRGS (excluding residues 1-42, which are not present in the crystal structure) was computed using the program GRASP (32). The surface of the protein presumed to interact with G$\alpha_{13}$ faces the viewer. Residues that were mutated are labeled. The surface corresponding to each
solvent accessible residue is color coded, from light pink to deep red, in proportion to the loss of GAP activity as measured by $%V_{0}^{WT}$ (Fig. 2C) upon mutation. The surfaces of residues that were not mutated, or where mutation had no significant affect on GAP activity ($%V_{0}^{WT} > 80\%$), are rendered in light gray.  

$B$, the corresponding surface representation of RGS4, color-coded according to GAP activity as in panel A, using relative activity data obtained for mutants of RGS4 reported by Srinivasa, et al., Table 1 (16). The activity data obtained in the presence of 200 nM RGS4 was used to color-code this surface diagram. Only those solvent accessible residues mutated to alanine were color-coded in for this diagram. In both panels, circles labeled and color-coded according to the scheme used in Fig. 1A, enclose structural elements that form the RGS4:G$\alpha_{i1}$ binding site, or the proposed p115rgRGS:G$\alpha_{13}$ binding site.

**FIG. 4.** *Effect of mutations within the amino terminus of p115rgRGS on GAP activity.*  

*A*, the time course of GTP hydrolysis by G$\alpha_{13}$, as described in Fig. 2A, is shown for the basal condition (*closed circles*), or inclusion of the 10 nM wild type (*open squares*), E32K mutant (*open circles*), E34K mutant (*closed triangles*), E27K mutant (*open triangles*) or E29K mutant (*closed squares*) forms of the p115rgRGS domain. *B*, the time course of GTP hydrolysis by G$\alpha_{13}$, as described in Fig. 2A, is shown for basal conditions (*closed circles*), or inclusion of the 10 nM wild type (*open squares*), S44A mutant (*open circles*), Q45A mutant (*closed triangles*), D28A mutant (*open triangles*) or F31A mutant (*closed squares*) forms of the p115rgRGS domain. *C*, the apparent initial rate of GTP hydrolysis catalyzed by G$\alpha_{13}$ in the presence of each p115rgRGS mutant (10 nM) is represented as a percentage of the initial rate of GTP hydrolysis by G$\alpha_{13}$ in the
presence of wild type p115rgRGS domain. D, the quantity of $^{32}$P$_i$ released in the assay described in panel A after two minutes of incubation was determined in the presence of the indicated concentration of mutant or wild type p115rgRGS: wild type p115rgRGS (closed squares), E27K (closed circles), D28A mutant (open circles), E29K mutant (closed triangles) or F31A mutant (open triangles).

FIG. 5. Binding of G$\alpha$13 to rgRGS domain mutants. Purified GST-tagged rgRGS domains (20 pmol) were bound to Glutathione Sepharose 4B (see “Materials and Methods”). Washed beads were incubated with gel-filtered GDP-bound G$\alpha$13 (20 pmol) prepared in the presence or absence of AMF (30 μM AlCl$_3$, 5 mM MgCl$_2$, and 5 mM NaF) and mixed gently for 30 minutes at 4 ºC. The beads were then washed and the relative amount of G$\alpha$13 that remained bound to the immobilized rgRGS domains was determined by immunoblot with B860 anti-G$\alpha$13 antiserum after separation by SDS-PAGE.

FIG. 6. GAP and G$\alpha$13 binding activities of p115RhoGEF containing mutations in the rgRGS domain. A, G$\alpha$13 was loaded with [γ-$^{32}$P]GTP (final concentration of 1-5 nM) and hydrolysis of the bound GTP was initiated as described in Fig. 2 either in the absence or presence of the indicated concentrations of wild type (WT) p115RhoGEF or the exchange factor containing the indicated mutations in its rgRGS domain: wild type (closed circles), D156A mutant (open circles), E27K mutant (closed triangles), D28A mutant (open triangles), E29K mutant (closed squares) or F31A mutant (open squares). Hydrolysis of GTP was measured for 2 min at 4 ºC. B, Binding of G$\alpha$13 to
the mutant proteins was done as described in Fig. 5 except the Glu-tagged p115RhoGEFs were bound to Sepharose containing immobilized anti-Glutag IgG.

Fig. 7. Stimulation of p115RhoGEF mutants by Go13. Exchange of guanine nucleotide on Rho was assessed by binding of [35S]GTPγS (see “Materials and Methods”) either in the absence or presence of p115RhoGEF and the addition of increasing concentrations of activated Go13 as indicated. Reactions contained 2 µM 6H-RhoA and 20 nM p115RhoGEF when indicated: wild type (open circles), F31A mutant (closed triangles), D28A (open triangles), D156A (closed squares), no GEF (closed circles). Reactions were incubated at 30 °C for 3 min. Activation of Go13 was accomplished by preincubation with AMF at 4 °C for 30 min and inclusion of AMF in the exchange assay.

FIG. 8. Putative Go13 binding site for the N-terminus of p115RhoGEF. A, the sequence of the N-terminal 41 residues of p115RhoGEF; acidic residues are colored red and basic residues colored blue. B, the complex of p115rgRGS (colored as in Fig. 1A) with Go13 (gray except for switch segments in purple) was modeled as described (26). The position of the most N-terminal residue that is defined in the structure of the p115rgRGS domain (residue 44) is labeled. C, the putative binding site for the N-terminus of p115RhoGEF (a detailed view of the area shown enclosed by a dotted box in panel B) is formed by the helical domain and the switch regions of Go13, and helix α8 of the rgRGS domain.
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Mapping the Gα13 binding interface of the rgRGS domain of p115RhoGEF
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