Chimeric Ga\textsubscript{12}/Ga\textsubscript{13} Proteins Reveal the Structural Requirements for the Binding and Activation of the RGS-like (RGL)-containing Rho Guanine Nucleotide Exchange Factors (GEFs) by Ga\textsubscript{13}*

Received for publication, September 14, 2004
Published, JBC Papers in Press, October 14, 2004, DOI 10.1074/jbc.M410594200

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The \(\alpha\)-subunit of G proteins of the G\textsubscript{12/13} family stimulate Rho by their direct binding to the RGS-like (RGL) domain of a family of Rho guanine nucleotide exchange factors (RGL-RhoGEFs) that includes PDZ-RhoGEF (PRG), p115RhoGEF, and LARG, thereby regulating cellular functions as diverse as shape and movement, gene expression, and normal and aberrant cell growth. The structural features determining the ability of Ga\textsubscript{12/13} to bind RGL domains and the mechanism by which this association results in the activation of RhoGEFs are still poorly understood. Here, we explored the structural requirements for the functional interaction between Ga\textsubscript{13} and RGL-RhoGEFs based on the structure of RGL domains and their similarity with the area by which RGS4 binds the switch region of Ga\textsubscript{1} proteins. Using Ga\textsubscript{13}, which does not bind RGL domains, as the backbone in which Ga\textsubscript{13} sequences were swapped or mutated, we observed that the switch region of Ga\textsubscript{13} is strictly necessary to bind PRG, and specific residues were identified that are critical for this association, likely by contributing to the binding surface. Surprisingly, the switch region of Ga\textsubscript{13} was not sufficient to bind RGL domains, but instead most of its GTPase domain is required. Furthermore, membrane localization of Ga\textsubscript{13} and chimeric Ga\textsubscript{12} proteins was also necessary for Rho activation. These findings revealed the structural features by which Ga\textsubscript{13} interacts with RGL domains and suggest that molecular interactions occurring at the level of the plasma membrane are required for the functional activation of the RGL-containing family of RhoGEFs.

Rho GTPases, which include Rho, Rac, and Cdc42, play a central role in the regulation of a number of basic cellular events such as cell movement and changes in cell shape, as well as in the control of gene expression regulation and cell growth (1). These GTP-binding proteins act as molecular switches that are inactive in their GDP-bound form, and upon exchange of GDP for GTP, they adopt an active conformation in which they can interact with their specific effector molecules, thereby affecting their localization and/or activity (1–3). This nucleotide exchange is promoted by a large family of guanine nucleotide exchange factors (GEFs),1 the vast majority of which are characterized by the presence of a dbl-homology (DH) and pleckstrin homology (PH) domain (2, 4). These GEFs also exhibit a number of additional regulatory regions by which they are strictly controlled by a diverse array of upstream signaling pathways, including those initiated by cell adhesion molecules, tyrosine kinase growth factor receptors, as well as by G protein-coupled receptors (GPCRs) (2, 5).

In particular for Rho, this GTPase participates in many physiological and pathological processes that involve the activation of GPCRs. For example, GPCRs such as those for thrombin and lysophosphatidic acid (LPA) promote cytoskeletal changes and expression from serum responsive element (SRE)-regulated genes by activating Rho (6, 7). Rho also participates in platelet aggregation (8) and in smooth muscle contraction when elicited by a large number of vasoactive hormones that act on GPCRs (9). The pathway by which these GPCRs stimulate Rho involves the activation of \(\alpha\)-subunits of the G\textsubscript{12/13} and G\textsubscript{4} family of heterotrimeric G proteins. Ga\textsubscript{12/13} in turn stimulate Rho through the direct interaction with a group of Rho GEFs characterized by the presence of a RGS-like (RGL) domain (10–12), whereas Ga\textsubscript{4} activates Rho through a still not fully understood mechanism (13, 14).

The family of RGL-containing Rho GEFs comprises three members: PDZ-RhoGEF (PRG) and LARG, which contain an N-terminal PDZ domain, and p115-RhoGEF (p115), which lacks this N-terminal protein-protein interaction domain (10–12). The PDZ domain of PRG and LARG mediates the interaction of these GEFs with membrane receptors including plexins of the B family and insulin-like growth factor receptor (15–18). The RGL domain is followed by DH and PH homology domains, by which they promote the nucleotide exchange on Rho, and a long C-terminal domain that harbors regulatory properties (19–21). The RGL domain is directly recognized by receptor-stimulated Ga\textsubscript{12/13}, thus providing a molecular bridge for the activation of Rho by Ga\textsubscript{12/13} (19), and in the case of p115, this domain also acts as a GTPase-activating protein (GAP) for Ga\textsubscript{13} (22). However, this GAP activity is not required to couple G\textsubscript{13} to Rho activation, as p115 mutants that possess a reduced GAP activity toward Ga\textsubscript{13} and a decreased binding affinity for this \(\alpha\)-subunit, still exhibit a normal ability to stimulate Rho

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1 The abbreviations used are: GEF, guanine nucleotide exchange factor; PH, pleckstrin homology; DH, dbl-homology; GPCR, G protein-coupled receptor; SRE, serum responsive element; GAP, GTPase-activating protein; RGL, RGS-like; HEK, human embryonic kidney.

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* This work was supported by National Institutes of Health Grant D43 TW06664, the Fogarty International Center, NIDCR, National Institutes of Health, and grants from CONACYT and Fundación Miguel Aleman. 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.

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exchange when activated by Go13 (23).

The Go subunit is composed of a α-helical domain and a GTPase domain, which includes a switch region that changes conformation upon nucleotide exchange, and is directly involved in binding and hydrolysis of GTP (24, 25). In the inactive heterotrimer the Go subunit, with GDP bound to it, keeps stable interactions with the heterodimer Goγ. Therefore, the effector motifs are covered in the heterotrimer, rendering both Go and Goγ unable to activate their respective effectors. Agonists acting on GPCRs induce a structural change that results in the nucleotide exchange and the dissociation of the heterotrimer, allowing the activation of Goα- and Goγ-dependent effectors. Signaling is terminated by the hydrolysis of GTP to GDP by virtue of the intrinsic GTPase activity of Go subunits, which is further stimulated by the interaction with regulators of G protein signaling (RGS proteins).

In this regard, the crystal structure of the complex formed by RGS4/Go (26), and that of the RGL domains of PRG and p115 (27, 28), have provided a model for the likely mechanism by which the activity of Go subunits is terminated, as well as the process by which Go13 and RGL-RhoGEFs might establish a functional interaction. Regarding the latter, in the proposed model the region including switch 1 and switch 2 within the GTPase domain of Go12/13, would be predicted to participate in the interactions with the RGL domain from RGL-RhoGEFs, whereas the N-terminal α-helical domain from Go12/13 would not establish direct contact interactions with them. As no structural information is yet available for Go12/13 members, we have addressed in this study the structural requirements for Go13-dependent stimulation of Rho by engineering chimeric G proteins using Go2, which does not activate Rho-RhoGEFs, as the backbone in which Go13 sequences were swapped or mutated. The emerging results revealed that the entire switch region from Go13 is necessary but insufficient to exert a Rho-stimulating activity when expressed in the context of Go2. In fact, Go13 depends on most of its GTPase domain, excluding its C-terminal 36 amino acids, for a functional interaction with PRG. Within this region, the integrity of both switch 1 and switch 2 is strictly required for a maximal effect. Furthermore, membrane localization of Go13 or the Rho-activating chimeric Goα subunits is also necessary for Rho activation, gene expression, and cell transformation. These findings indicate that specific structural features present in Go13 together with additional molecular interactions occurring at the level of the plasma membrane are required for the effective coupling of Go13 to the GTPase containing family of RhoGEFs, and ultimately to stimulate Rho.

MATERIALS AND METHODS
Bioinformatic Tools—The structure of Go13/RGS4 (26) was analyzed with the CNS3D program (www.ncbi.nlm.nih.gov/Structure/CNS3D/ CNS3D.shtml) to identify amino acids in the Go13-RGS4-contact interface within 3 Å. Sequence alignment corresponding to the switch region from representative members of each Go protein family was performed using ClustalW (www.ch.embnet.org/software/ClustalW.html) and the figure prepared with Boxshade (www.ch.embnet.org/software/BOX_form.html).

DNA Constructs—The cDNAs for Goα, Go2, and human Go13, GTPase-deficient mutants as templates, and cloned into the mammalian expression vector pcDNA2 by EcoRI and XbaI restriction sites that were introduced with the 5′- and 3′-primers, respectively. The cDNAs corresponding to the N-terminal, switch, and C-terminal regions were first obtained by PCR in which the internal primers were designed to overlap with the sequence of the cDNA of the fragments to be fused with in the second PCR reaction. The corresponding chimeric cDNAs were obtained by a second PCR reaction in which the three initial fragments were mixed as templates. The second set of chimeras in which the content of Go13 was extended from the switch region toward the extremes was also obtained by PCR, in this case the template for the second PCR was the mixture of two cDNA fragments corresponding to either N- or C-terminal domains, as indicated in the respective figures. From this second series, the chimeras in which the content of Go13 extended from the switch region toward the C-terminal end was used as template for further modifications, which included reduction of the contribution of Go13 at the C-terminal end by substitution with the corresponding sequences from Go2, and point mutations at the putative RGL-contact sites within the switch region and at the N-terminal myristylation signal. Point mutations were performed either by PCR to mutate the N-terminal myristylation signal by substituting Gly for Ala in the second codon, and by the QuikChange site-directed mutagenesis kit from Stratagene to substitute the putative RGL-contact sites, following the manufacturer’s instructions. All chimeric and mutant molecules were sequenced at the NIDCR DNA sequencing facility, and their expression was confirmed by Western blot using antibodies detecting the corresponding N-terminal domain. The sequence for the different primers will be made available upon request.

Cell Lines and Transfections—Human embryonic kidney (HEK) 293T cells were maintained in Dulbeco’s modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum. For transient transfections, tissue culture plates were treated for 10 min with phosphate-buffered saline containing 5 μg/ml poly-o-lysine before seeding the cells to prevent them from detaching from the plates during the transfection procedure and thereafter. Transient transfections in HEK 293T cells were performed using the Lipofectamine (Invitrogen) according to the manufacturer’s instructions. NIH 3T3 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) calf serum, and were used to monitor the transforming potential of the different chimeras as described (7).

Western Blots and Protein-Protein Interactions—Transfected cells were lysed at 4 °C in a buffer containing 50 mM Tris, pH 7.4, 0.15 μM NaCl, 0.1% Triton X-100, 20 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin, and insoluble material was removed by centrifugation. Lysates containing ~50 μg of total cellular protein or affinity isolated proteins (see below) were analyzed by Western blotting after SDS-polyacrylamide gel electrophoresis and visualized by enhanced chemiluminescence detection (Amersham Biosciences) using rabbit anti-Gα13 (Santa Cruz Biotechnology) or rabbit antibody to Go12/13 (Santa Cruz Biotechnology) depending on whether the N-terminal domain of the transfected G protein chimeras was from Goα13, or goat anti-rabbit (Cappel) IgGs coupled to horseradish peroxidase as a secondary antibody. To test the ability of the different chimeras to interact with RGL domain from PRG, lysates from HEK 293T cells were incubated with bacterially expressed six histidine-tagged RhoGEF C-terminal RGL isolated with talon beads (Clontech) as previously described (27).

Luciferase Assays—Cells in 24-well plates were transfected with different expression plasmids together with 0.1 μg of pSRE luciferase reporter plasmid, pNull Renilla, and pcDNAIII-His-gal (a plasmid expressing β-galactosidase) to normalize for transfection efficiency. Firefly and Renilla luciferase were present in cells co-transfected using a dual-luciferase reporter system (Promega), and light emission was quantitated using a Monolight 2010 luminometer (Analytical Luminescence Laboratory) as specified by the manufacturer (39).

In Vivo Rho Activation Assay—HEK 293T cells were transfected using the Lipofectamine Plus™ reagent. The day after transfection, cells were cultured for 24 h in serum-free Dulbecco’s modified Eagle’s medium and assayed for Rho activity using the Rho-binding domain (RBD) of rhokin in 10-cm dishes were transfected with the indicated chimeras and grown for 48 h. Cells were washed once with phosphate-buffered saline and then dislodged from the plate by washing and pelleted at low speed. The cell pellet was suspended in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 8, 2 mM MgCl2, 1 mM EDTA, 1 mM diethiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin), and cells were lysed

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by 10 passages through a 27-gauge needle. Lysed cells were centrifuged at 2500 rpm for 5 min at 4 °C to remove nuclei and intact cells. The supernatant was centrifuged at 14,000 rpm for 80 min at 4 °C, and the pellet (particulate fraction) was suspended in an equal volume of lysis buffer. For the detection of RhoA a monoclonal antibody was used as indicated in “Materials and Methods.” The control included cells transfected with empty vector. The different chimeric G proteins were identified with an antibody recognizing the N-terminal domain as indicated on the left.

FIG. 1. The switch region from Gα13 is required but not sufficient to promote Gα13-dependent activation of Rho and PRG 13-dependent Rho Activation. A, structure of the Gα13/RGS4 complex (26), on the right panel the structure of RGS4, shown as dark gray on the left, was omitted, and the location of RGS4-contacts within 3 A, as detected with the NCBI CN3D program, is shown. B, schematic representation of GTPase-deficient chimeric Gα12/Gα13, constructs where the switch region, including the putative RGS- or RGL-interacting determinants, was swapped. The lower panel shows the alignment of the switch region from representative members of the different families of Gα subunits. Arrows indicate the amino acids intervening in the RGS4-G protein contact interface, numbered as in A, in switch 1 to switch 3, which are indicated with a thick line. The region that was swapped extends 25 amino acids toward the C-terminal end. C, expression and activity of GTPase-deficient Gα12, Gα13, and chimeric G proteins represented in B. The graph in the upper part shows the luciferase reporter for Rho-dependent SRE activity of HEK 293T cells transfected with the indicated chimeras. Their expression in total cell extracts (TCE) and ability to interact with the RGL domain from PRG (AP RGL) and to promote the activation of endogenous RhoA (AP GST) is shown. The content of Rho in total cell extracts (TCE) is shown at the bottom. The different chimeric G proteins were identified with an antibody recognizing the N-terminal domain as indicated on the left. For the detection of RhoA a monoclonal antibody was used as indicated in “Materials and Methods.” The control included cells transfected with empty vector.

RGL-RhoGEFs might be similar to that of the complex formed by Gα1 and RGS4 shown in Fig. 1A (26). Residues in Gα1 that provide surface areas in direct contact with RGS4 are indicated. In particular, this region can be subdivided into 3 switch areas, which are highly conserved among each G protein α-subunit family. Of interest, all 6 residues involved in the interaction between Gα1 and RGS4 are also conserved in Gα12 and Gα13, but 3 of them are quite distinct in Gα11 and Gα13, as depicted in Fig. 1B. Nonetheless, as no information regarding the three-dimensional characteristics of Gα13 family members is available, the molecular basis of their interaction with RGL-RhoGEFs and the consequent activation of Rho remain to be determined. To begin addressing this issue, we first evaluated the possibility that the switch region from Gα13 contains all the structural elements required to elicit Rho activation. For this purpose, we engineered G protein α-subunits in which the switch region from active Gα13, Gα13QL, replaced the corresponding region from active Gα12, Gα12QL (Fig. 1B). The resulting chimera, named Gα12SWα13QL, contains the first 194 amino acids (Met1-Gln194) from Gα12 and Gα13, as depicted in Fig. 1B. The reciprocal chimera, Gα13SWα12QL, contains the first 194 amino acids (Met1-Gln194) from Gα13 followed by a central part (Asp195-Ile287), including the switch region from Gα12QL, and the C-terminal (Leu267-Phe355) from Gα12, as indicated. The possibility that the functional interaction between Gα12/13 and RGL-RhoGEFs might be similar to that of the complex formed by Gα1 and RGS4 shown in Fig. 1A (26). Residues in Gα1 that provide surface areas in direct contact with RGS4 are indicated. In particular, this region can be subdivided into 3 switch areas, which are highly conserved among each G protein α-subunit family. Of interest, all 6 residues involved in the interaction between Gα1 and RGS4 are also conserved in Gα12 and Gα13, but 3 of them are quite distinct in Gα11 and Gα13, as depicted in Fig. 1B. Nonetheless, as no information regarding the three-dimensional characteristics of Gα13 family members is available, the molecular basis of their interaction with RGL-RhoGEFs and the consequent activation of Rho remain to be determined. To begin addressing this issue, we first evaluated the possibility that the switch region from Gα13 contains all the structural elements required to elicit Rho activation. For this purpose, we engineered G protein α-subunits in which the switch region from active Gα13, Gα13QL, replaced the corresponding region from active Gα12, Gα12QL (Fig. 1B). The resulting chimera, named Gα12SWα13QL, contains the first 194 amino acids (Met1-Gln194) from Gα12 followed by a central part (Asp195-Ile287), including the switch region from Gα12QL, and the C-terminal (Leu267-Phe355) from Gα12, as indicated. The reciprocal chimera, Gα13SWα12QL, contains the first 194 amino acids (Met1-Gln194) from Gα13 followed by a central part (Asp195-Ile287), including the switch regions, from Gα12Q205L,
and the C terminus (Leu288-Gln377) from Go13. The activity of these chimeras was determined by their ability to stimulate a mutant SRE that responds to Rho (7), and by their ability to interact with the RGL domain of PRG and to stimulate Rho. The different chimeric G proteins were detected by antibodies recognizing the N-terminal domain of either Go12 or Go13 (Fig. 1C). As expected, a GTPase-deficient Go13 strongly stimulated Rho-dependent pathways, while active Go12 was unable to do so. However, surprisingly, a chimeric Go2 containing what was expected to be the critical region for the interaction with RGL-RhoGEFs, did not gain the ability to stimulate Rho or to interact with PRG (Go2SWo13QL, Fig. 1C). Go13 on the other hand, lost these properties when its switch region was replaced by that of Go2 (Go12SWo13QL, Fig. 1C). Direct evaluation of Rho-GTP content in transfected HEK-293T cells indicated that swapping the switches from Go13 into Go12 provided to the resulting chimera only a very limited ability to stimulate Rho, while the reciprocal chimera was unable to do so (Fig. 1C, lower panel).

The Structural Requirements for Rho Activation by Go13 Extend toward the C-terminal Domain of Go13—As swapping the switch region from Go13QL into Go2QL was not sufficient to confer to Go2 the ability to stimulate Rho, we predicted that additional structural elements from Go13 were required to support this effect. In order to test this possibility, we engineered additional chimeras where the contribution from Go13QL into the Go12 backbone was extended from the switch region toward their N or C termini (Fig. 2, upper panel). Chimeras in which only the N- or C-terminal domains were swapped were also tested as controls. The expression of each chimera was confirmed by Western blotting (Fig. 2, lower panel). By this approach, we found that a chimeric G protein (Go12SW-Co13QL), that includes the switch region and the C-terminal domain, was able to stimulate Rho-dependent luciferase reporter gene comparable to that of the GTPase-deficient Go12QL (Fig. 2, lower panel).

The Active Go12SW-Ca13QL Chimera Requires the Integrity of the Switch Region and an Adjacent C-terminal Extension.—To narrow down the minimal structural requirements for Go13QL to stimulate Rho, the active chimera (Go12SW-Co13QL) was further modified by replacing additional sequences for those from Go13 corresponding to each of the switch subregions, or the C-terminal domain, as indicated in Fig. 3, upper panel. The expression of this set of chimeras was demonstrated by Western blot (Fig. 3, lower panel). When either switch 1 or switch 2 and 1 in Go12SW-Ca12QL were substituted by those from Go13, the resulting chimeras, Go12SW2,3-Co13QL and Go13QLSW3-Co13, respectively, were unable to stimulate Rho-dependent pathways (Fig. 3, lower panel). On the other hand, the contribution from Go13 at the C-terminal end of Go12SW-Ca13QL was narrowed down by substituting it for sequences from Go12 as indicated in Fig. 3, upper panel. Based on this approach, we observed that the last 36 amino acids in Go12SW-Ca13QL (Pro320-Gln356) could be replaced with those from Go12 with no reduction in the Rho-stimulating activity of the resulting chimera, Go12SW-320a13QL, whereas a further reduction, replacing the last 61 amino-acids (Pro305-Gln366), resulted in a chimeric G protein, Go12SW-295a13QL, that was unable to activate Rho. Go12SW-320a13QL was therefore referred to herein as the “minimal active chimera” (Fig. 3, lower panel).

Point Mutations on the Putative RGL Contact Sites within Switch 1 and 2 of the Minimal Active Chimera Go12SW-320a13QL Reduce Its Activity—Based on the structure shown in Fig. 1A (26), amino acids from Go12 switch 1 and switch 2 region that were predicted to make contact with RGS4 were re-introduced by site-directed mutagenesis into the equivalent positions in Go12SW-320a13QL (Fig. 4, upper panel) (SW1 and SW2 mutants). The expression of these mutants was demonstrated by Western blot (Fig. 4, lower panel). Furthermore, as shown in Fig. 4, lower panel, Go12SW-320a13QL harboring mutations in the putative RGL contact sites in switch 1 or switch 2 exhibited a reduced ability to stimulate the SRE, which was nearly half of that of Go12SW-320a13QL, while the double mutant showed no activity. The ability of these chimeras to interact with PRG and to stimulate the increase in GTP-Rho was aligned with their activity detected in the luciferase reporter assay as shown in Fig. 4, lower panel.

An N-terminal Lipid Modification in the Go12SW-320a13QL Chimera Is Not Required for Binding to PRG, but Is Necessary for a Functional Interaction Leading to the Activation of Rho—The minimal Go12SW-320a13QL active chimera possesses a Go12 N-terminal region that exhibits a myristylation signal instead of the palmitoylation signal known to be present in Go13 (29). Thus, we tested if this myristylation signal had any influence on the ability of this chimera to interact with PRG. A Go12SW-320a13QL mutant chimera (Go12G2A-SW-320a13QL), in which the myristylation signal was mutated, was well expressed (Fig. 5B, lower panel) but exhibited a strongly reduced ability to interact with the plasma membrane as judged by

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**FIG. 2. Go12QL-structural requirements for Go13-dependent activation of Rho extend from the switch region toward the C-terminal end of chimeric Go12Go13 proteins.** Upper panel, schematic representation of GTPase-deficient chimeric Go12Go13 constructs. Switch region is indicated. The graph in the middle panel shows the luciferase reporter for Rho-dependent SRE activity of HEK 293T cells transfected with the indicated chimeras. The expression of the different GTPase-deficient Go12, Go13, and chimeric G proteins represented at the top was detected by Western blot in total cell extracts (TCE). The control included cells transfected with empty vector.
membrane fractionation (Fig. 5A, lower panel). Interestingly, this mutant was still able to interact with PRG, supporting that a stable interaction between Ga13 and PRG does not require additional, membrane-derived elements. However, neither this mutant Ga12 chimera nor a mutant Ga13 lacking palmitylation sites stimulated the accumulation of GTP-Rho (Fig. 5B, lower panel and data not shown). Thus, the interaction between Ga13 and PRG is required but not sufficient for the activation of Rho. Indeed, membrane association or the subsequent interaction with membrane-associated molecules might be also required to induce the GEF activity of the Ga13-RGL-containing GEF complex.

**Ga13-SW-320AQ13QL Induces Cell Transformation**—In order to examine whether the structural features providing the ability to activate Rho were related to those determining the transforming potential of Ga13, we tested the focus-forming activity of the chimeras and point mutants derived from the minimal active chimera to induce focus formation. As shown in Fig. 6, the efficiency of Ga13-SW-320AQ13QL and its mutants to induce transformation of NIH 3T3 fibroblasts correlated nicely with its ability to stimulate Rho (see above). On the other hand, as expected, the myristylation-deficient Ga12G2A-SW-320AQ13QL chimera was not transforming (results not shown).

**DISCUSSION**

In this report we have investigated the nature of the structural elements required for the functional interaction between Ga13 and RGL-containing RhoGEFs. We took advantage of the fact that members of the Ga family of heterotrimeric G protein subunits do not bind to PRG and cannot stimulate Rho and its downstream pathways to use the primary sequence of Ga13 as the backbone in which Ga13 sequences were swapped. Our findings indicate that the switch region within the GTPase domain of Ga13 is necessary for the activation of Rho, and within this
region, two residues in switch 1 and switch 2 were identified that are strictly required for this function. However, neither of these residues, which act in an additive fashion, nor the entire switch region are alone sufficient to exert a Rho-stimulating activity when introduced into Ga12. Indeed, additional structural elements located toward the C terminus of Ga13 are required to bind PRG, whereas lipid modification, and hence membrane localization of Ga12 or chimeric Ga12 subunits, is necessary for Rho-acting upon binding to RGL-containing GEFs.

The crystal structure of the complexes formed by RGS4 with Ga13 (26) and RGS9 with Ga13 (30) revealed that switch 1 and switch 2 within the Ga13-GTPase domain harbors critical interacting residues at the interface between the Ga subunit and the RGS protein. Considering the structural similarity between the recently reported structures of the RGL domains from PRG and p115 and the RGS domain of RGS4 and RGS9, it would be expected that Ga13 might use similar elements to bind RGL-containing GEFs as those employed by Ga12 or Ga13 for their respective complexes with these RGS proteins (27, 28). If so, the replacement of the three switches in Ga12-QL for those of Ga13-QL would be expected to result in a G protein α-subunit incapable of activating Rho. Conversely, the presence of the switch region from Ga12-QL would be expected to confer Ga12-QL the ability to stimulate Rho. Our results indicate that the first premise is correct while the second is not, as additional elements toward the C-terminal end of the Ga13-GTPase domain are required to activate Rho and its downstream pathways.

Regarding the contribution of the Ga13 switch region, the structure of RGS4 bound to Ga12 revealed that threonine 182, present in switch 1 of Ga12, exhibits the strongest interaction with this RGS (26). This residue is conserved among all G protein α-subunits but in Ga12 and Ga13, which exhibit a lysine in this particular position. Similarly, histidine 213 in switch 2 of Ga13 is part of the contact surface with RGS4, and this residue is also conserved among all G protein α-subunits with the exception of Ga12/13. To analyze whether these residues in Ga13 participate in binding to RGL-RhoGEFs, we mutated them together with adjacent non-conserved residues for those corresponding to Ga12 in the minimal fully functional Ga12/13 chimeras. When these putative contact sites in either switch 1 or switch 2 were mutated, a reduced ability to stimulate Rho was observed that was abolished when mutations in both switch regions were introduced simultaneously. These findings indicated that these residues in switch 1 and switch 2 are strictly required to interact with RGL-containing GEFs, and are likely to contribute to the contact interface between Ga12/13 and the RGL-like domain in an additive fashion. This observation may help explain why α-subunits of the G13 family fail to bind PRG and other RGL-containing GEFs (11, 12), and, conversely, why Ga12 and Ga13 may not bind to and interact with RGS4 and other related RGSs (26, 27, 28).
31). Thus, these particular residues within switch 1 and 2 may determine the choice of effector molecules and GAPs available to each G protein family, a key event for achieving signal specificity upon GPCR activation.

On the other hand, the need of sequences in addition to the switch region from $\alpha_{13}$ to stimulate Rho, suggests that either the overall structure of the GTPase domain of $\alpha_{13}$ may differ from that of $\alpha_1$, and thus its C-terminal region may be required for the correct spatial positioning of the residues present in the switch region, or that the overall structures of $\alpha_1$ and $\alpha_{13}$ might be indeed similar, but that additional interactions may exist beyond those involving the switch region of $\alpha_{13}$ that may be required for the functional coupling of this G protein to the membrane.

In conclusion, our results indicate that the structural requirement for RGL-dependent GEF family to the membrane is a major determinant of the ability of $\alpha_{12/13}$ to stimulate Rho. Indeed, whereas the N-terminal $\alpha$-helical domain does not provide structural elements for the activation of Rho, the lipid modification is needed to bring the chimeric $\alpha_{12/13}$ G protein to the membrane in order to be active. In this regard, the fact that it does not appear to exist a preferential lipid modification on the $\alpha_2$ protein, as myristylation of $\alpha_2$ (32, 33) can functionally replace the palmitylation of $\alpha_{12/13}$ to induce cell transformation (34) and to stimulate Rho, and each target preferentially a distinct membrane compartment (35, 36), this process is unlikely to involve the interaction of RGL-containing GEFs with additional molecules exhibiting restricted membrane subdomain distribution. Instead, membrane recruitment through the RGL domain may enhance the local concentration of these RhoGEFs at the inner face of the plasma membrane. These GEFs may then interact through other regions with membrane components, such as membrane phospholipids through their PH domain, thus either causing a conformational change in the DH domain that then becomes activated, or adopting an orientation with respect to the lipid bilayer that facilitates the interaction of the DH domain with membrane-bound Rho. This membrane interaction might be also required for the regulatory properties of tyrosine or serine/threonine protein kinases that modulate RGL-RhoGEFs, such as focal adhesion kinase and PAK4 (21, 37), respectively. These, as well as other possibilities are under current investigation.

In conclusion, our results indicate that the structural requirements for the functional interaction between $\alpha_{13}$ and RGL-RhoGEFs involve a large fraction of the GTPase domain of $\alpha_{13}$ that includes the switch region, and in particular two conserved residues within switch 1 and 2 that act in an additive fashion, a C-terminal extension, and the N-terminal domain of $\alpha_{13}$, which does not participate in binding to these GEFs but is strictly required to stimulate Rho and cell transformation.
due to its membrane targeting lipid modification. As increasing interest on the structural characteristics of RGS-G protein interactions has emerged from the potential use of small molecule inhibitors that may block or regulate such interactions (38), we can envision that the present findings defining the structural determinants required for the coupling of Gα13 to Rgl-RhoGEFs and Rho activation may facilitate the identification of novel therapeutic approaches for the treatment of the numerous human diseases that are dependent on the dysfunction of GPCRs that act on Gα13 to stimulate Rho.

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J. Biol. Chem. 2004, 279:54283-54290.
doi: 10.1074/jbc.M410594200 originally published online October 14, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410594200

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