Determinants at the N- and C-termini of Gα₁₂ required for activation of Rho-mediated signaling

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Abstract

Background: Heterotrimeric guanine nucleotide binding proteins of the G12/13 subfamily, which includes the α-subunits Gα₁₂ and Gα₁₃, stimulate the monomeric G protein RhoA through interaction with a distinct subset of Rho-specific guanine nucleotide exchange factors (RhoGEFs). The structural features that mediate interaction between Gα₁₃ and RhoGEFs have been examined in crystallographic studies of the purified complex, whereas a Gα₁₂:RhoGEF complex has not been reported. Several signaling responses and effector interactions appear unique to Gα₁₂ or Gα₁₃, despite their similarity in amino acid sequence.

Methods: To comprehensively examine Gα₁₂ for regions involved in RhoGEF interaction, we screened a panel of Gα₁₂ cassette substitution mutants for binding to leukemia-associated RhoGEF (LARG) and for activation of serum response element mediated transcription.

Results: We identified several cassette substitutions that disrupt Gα₁₂ binding to LARG and the related p115RhoGEF. These Gα₁₂ mutants also were impaired in activating serum response element mediated signaling, a Rho-dependent response. Most of these mutants matched corresponding regions of Gα₁₃ reported to contact p115RhoGEF, but unexpectedly, several RhoGEF-uncoupling mutations were found within the N- and C-terminal regions of Gα₁₂. Trypsin protection assays revealed several mutants in these regions as retaining conformational activation. In addition, charge substitutions near the Gα₁₂ N-terminus selectively disrupted binding to LARG but not p115RhoGEF.

Conclusions: Several structural aspects of the Gα₁₂:RhoGEF interface differ from the reported Gα₁₃:RhoGEF complex, particularly determinants within the C-terminal α5 helix and structurally uncharacterized N-terminus of Gα₁₂. Furthermore, key residues at the Gα₁₂ N-terminus may confer selectivity for LARG as a downstream effector.

Keywords: Gα₁₂, Gα₁₃, Heterotrimeric G protein, RhoGEF, Rho, LARG, Serum response element

Background

The G12/13 subfamily of heterotrimeric guanine nucleotide binding proteins (G proteins) is comprised of two α-subunits in mammals, Gα₁₂ and Gα₁₃, that have been implicated in a variety of physiological and pathological cellular responses that include proliferation, cytoskeletal rearrangements, migration, and metastatic invasion [1,2]. A diverse set of putative effector proteins have been identified as direct interactors with one or both G12/13 subfamily members; however, the roles of individual Ga-effector interactions in specific cellular responses remain largely undefined [3]. The most extensively characterized G12/13 target proteins are a subset of Rho-specific guanine nucleotide exchange factors (RhoGEFs) that activate the monomeric G protein Rho via tandem Dbl-homology/pleckstrin-homology domains [4]. The Rho monomeric GTPases are known primarily for their role in regulating actin cytoskeletal dynamics, but these proteins also mediate cell polarity, microtubule dynamics, membrane transport pathways, transcription factor activity, cell growth, and tumorigenesis [5]. The G12/13-RhoGEF-Rho axis mediates critical signaling and developmental pathways in model organisms that include Drosophila melanogaster [6], Caenorhabditis elegans [7], and zebrafish [8]. In addition, direct interaction with RhoGEFs is required for mutationally activated Gα₁₂ to trigger increased invasiveness of breast cancer cells [9].

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Activated G12/13 α-subunits trigger Rho activation via binding and stimulation of three distinct RhoGEFs: p115RhoGEF, LARG and PDZ-RhoGEF [10-13]. This interaction is mediated primarily by a domain, located near the N-terminus of each RhoGEF, that is closely related to the regulator of G protein signaling (RGS) domain that defines the growing family of RGS proteins [14,15]. Although p115RhoGEF, LARG and PDZ-RhoGEF are highly similar in this “RGS homology” (RH) domain [16], these proteins appear to be activated by different mechanisms and play non-redundant roles in G12/13 subfamily-mediated signaling. Purified p115RhoGEF binds Goα12 and Goα13 and accelerates GTPase activity for both proteins, but only Goα13 can stimulate p115RhoGEF to activate RhoA in vitro [10,17]. Interaction of Goα12 or Goα13 with purified LARG can trigger its activation of RhoA; however, stimulation by Goα12 requires prior phosphorylation of LARG by the nonreceptor tyrosine kinase Tec [13]. Furthermore, studies utilizing small interfering RNA to hinder expression of specific RhoGEFs show that LARG is a specific downstream effector of thrombin receptor-mediated signaling, whereas signaling through the lysophosphatidic acid (LPA) receptor is attenuated by blocking PDZ-RhoGEF expression [18]. These results are compelling in light of a separate report that the thrombin receptor shows preferential coupling to Goα12, whereas the LPA receptor preferentially utilizes Goα13 as a conduit to downstream signaling [19]. Although it is possible that Goα12 stimulates a post-translationally modified form of p115RhoGEF or PDZ-RhoGEF in cells, the evidence to date suggests LARG as the most likely RhoGEF serving as a physiological effector for Goα12. Gains in our understanding of the specificity of RhoGEF engagement within the G12/13 subfamily should provide insights into the non-overlapping functions of Goα12 and Goα13 in signal transduction.

Crystallographic studies have revealed important structural aspects of the interaction between Goα13 and the RH domain of p115RhoGEF, including numerous residues in both proteins that provide contact points [20,21]. Initially, purification of Goα13 for crystallography required that it be engineered as a chimera in which amino acid sequence within several regions, including the N- and C-termini, was replaced by corresponding sequence from the Gi subfamily protein Ga1 [20]. The structure of the Goα13:p115RhoGEF-RH complex was later refined in crystallographic studies that utilized a Goα13 chimera harboring Ga1 sequence only at the N-terminus. Because the Goα N-terminus was unstructured in this crystallized complex, any role of this region in RhoGEF interaction remains to be determined. Although the region of Goα13 downstream of the Switch III region harbors several residues critical for RhoGEF engagement, notably Glu273, Thr274, Asn278, and Arg279 within the α3 helix and α3-β5 loop, other regions closer to the Goα13 C-terminus do not emerge in the crystal structure as providing key RhoGEF contact points [21].

In contrast to Goα13, a structure of Goα12 in complex with a RhoGEF target has not been reported, although a chimeric Goα12 harboring the N-terminus of Ga1 has been crystallized [22]. To examine the full sequence of Goα12 for structural features mediating its interaction with RhoGEFs, we engineered a series of cassette substitutions within constitutively activated Goα12 and examined these variants for in vitro binding to the RH domains of LARG and p115RhoGEF, as well as ability to drive the Rho-dependent process of serum response element (SRE) mediated transcription in cells [23]. Our results reveal unexpected regions of Goα12 as harboring determinants of its functional interaction with RhoGEFs, and also identify key charged amino acids near the Goα12 N-terminus that may confer selective binding to LARG.

Results

Myc-tagged Goα12 retains RhoGEF binding, Rho-mediated signaling, and conformational activation

To identify mutants of Goα12 impaired in RhoGEF binding, we first sought to establish an in vitro system in which Goα12 mutants could be expressed ectopically in cultured cells, rendered soluble in a detergent extract, and detected without interference from endogenous Goα12. We engineered the constitutively active Gln229-Leu variant of Goα12 (Goα12QL) to harbor a myc epitope tag, flanked by linkers of the sequence SGGGGS and positioned between residues Pro130 and Val140. This insertion site was chosen due to its approximate alignment with the position of green fluorescent protein in Gaq in a prior study [24]. We expressed myc-tagged and untagged Goα12QL in HEK293 cells, prepared detergent-soluble extracts, and analyzed these by immunoblotting. As shown in Figure 1A, myc-tagged Goα12QL was detected by both anti-myc and anti-Ga13 antibodies, with the latter generating a much stronger signal while avoiding an off-target 37 kDa band detected in all samples by the anti-myc antibody. Also, the myc-tagged protein (~45 kDa) was readily discernible from endogenous Goα12 and untagged Goα12QL (~43 kDa). Next, we subjected myc-Goα12QL to pulldown experiments using an immobilized GST fusion of the p115RhoGEF RH domain, as described in Methods. Myc-tagged and untagged Goα12QL bound to p115-RH with similar affinity (Figure 1B), and comparison with mock-transfected cells indicated the ~45 kDa band detected by anti-Goα12 was dependent on transfection with the myc-Goα12QL plasmid. Furthermore, LARG-RH and p115-RH showed similar ability to co-precipitate myc-tagged Goα12QL (Figure 1C). To ascertain that myc-Goα13 is functional as a mediator of cellular signal transduction through Rho, we measured transcriptional activation of a luciferase reporter gene positioned downstream of the serum response
element (SRE), a component of the c-fos promoter that provides a readout of Ga_{12}-mediated Rho activation [23]. Myc-tagged and untagged Ga_{12}^{QL} exhibited similar ability to stimulate this response in HEK293 cells co-transfected with SRE-luciferase (Figure 1D). Furthermore, trypsin digestion of HEK293 cell lysates harboring myc-Ga_{12}^{QL} yielded a protected fragment of ~40 kDa, comparable to results observed previously with GTPγS-loaded, purified Ga_{12}^{QL} [25]. An inactive, constitutively GDP-bound (Gly228Ala) variant of myc-tagged Ga_{12} did not yield this ~40 kDa fragment when digested with trypsin (Figure 1E). Taken together, these results suggest myc-Ga_{12}^{QL} undergoes conformational activation and retains normal signaling through the RhoGEF/Rho pathway. Because of the superior sensitivity of anti-Ga_{12} antibody in detecting myc-Ga_{12}^{QL}, and the easily discernable gel shift of Ga_{12} caused by the myc tag and linkers (see Figures 1A and B), we chose to utilize anti-Ga_{12} to detect myc-Ga_{12}^{QL} in subsequent protein binding experiments.

Mutations that uncouple Ga_{12} from RhoGEF binding and Rho-mediated signaling
To scan Ga_{12} for regions participating in its interaction with RhoGEFs, we utilized a comprehensive panel of

![Figure 1](http://www.jmolecularsignaling.com/content/8/1/3)
mutants in which sextets of consecutive amino acids in myc-Go\textsubscript{12}\textsuperscript{QL} are replaced by the sextet Asn-Ala-Ala-Ile-Arg-Ser (Figure 2 shows the native amino acid sextet and alphabetical designation for each mutant). This strategy of ”NAAIRS” cassette substitutions was chosen due to prediction of this motif being tolerated in the three-dimensional structure of proteins [26], prior use of this approach in mapping functional regions of both retinoblastoma and the telomerase catalytic subunit [27,28], and our previous success employing this strategy to identify Ga\textsubscript{12} determinants of binding to the scaffolding subunit of protein phosphatase-2A and the cytoplasmic tail of polycystin-1 [29,30]. Variants of Ga\textsubscript{12} were expressed in HEK293 cells and tested for interaction with immobilized LARG-RH, as described in Methods. As shown in Figure 3, myc-Go\textsubscript{12} was co-precipitated by a GST fusion of LARG-RH but not by GST alone. Many of these cassette mutants yielded a moderate-to-robust signal in the LARG-precipitated fraction; however, a subset displayed a weak or absent signal (Figure 3). To assess impairment of LARG binding for each myc-Go\textsubscript{12}\textsuperscript{QL} variant, we quantified the band intensity for each precipitated sample (pulldown), and divided this by the band intensity in the starting cellular extract (load). These calculations generated a ”pulldown:load ratio” for each mutant, and also for the positive control myc-Go\textsubscript{12}\textsuperscript{QL} that was tested in each experiment. Nearly all cassette mutants were solubilized by our detergent conditions and detected by immunoblotting; exceptions were mutant W, which we did not engineer due to overlap with the insertion site of the myc tag (see Figure 2), and mutant CC due to low expression levels that

**Figure 2** Residues replaced in Ga\textsubscript{12} cassette mutants. For each mutant, designated in italics (A-Z, AA-ZZ, AAA-KKK), the native amino acid sextet replaced by the sequence Asn-Ala-Ala-Ile-Arg-Ser is shown. An arrow between Pro\textsuperscript{139} and Val\textsuperscript{140} indicates the site of myc tag insertion. Mutant W was not produced. The dashed box indicates the native Glu\textsuperscript{229} mutated to Leu to render Ga\textsubscript{12} constitutively active. The native residues replaced in mutant KKK are Lys-Asp-Ile-Leu-Gln and thus partially overlap with mutant JJ. All cassette mutants contain the activating Q\textsuperscript{229}L mutation, except mutant LL due to its cassette substitution.
produced inconclusive results (data not shown). As shown in Table 1, the majority of cassette mutants exhibited pulldown:load ratios greater than 40% of the ratio determined for myc-Gα12QL as a positive control, using equal amounts of GST-LARG-RH (LARG) immobilized on glutathione-sepharose. Immobilized GST was utilized in parallel as a negative control. For all experimental samples, 20% of the volume was analyzed by SDS-PAGE and Coomassie blue staining to verify equal amounts of GST-LARG-RH and GST proteins in the precipitates (data not shown). Immunoblots displayed in this figure are representative of at least three trials per cassette mutant, except for mutants A-D, F-H, V, and KKK that showed minimal impairment in LARG binding after two trials. (Inset) Coomassie blue analysis of GST-fusion constructs expressed in bacteria and immobilized on glutathione-sepharose: GST-LARG-RH (LARG), GST-p115-RH (p115), and GST alone. Molecular weight standards (in kDa) are indicated at right.

Table 1  Gα12 cassette mutants impaired in binding LARG-RH

| Cassette substitution mutants of myc-Gα12QL (see Figure 2 for alphabetical designations) were expressed in HEK293 cells and subjected to protein interaction assays using a GST-fusion of the RH domain of LARG as described in Methods, and for each mutant a pulldown:load ratio was determined and calculated as a percent (left column) of the same ratio for unmodified myc-Gα12QL assayed in parallel. Each Gα12 mutant was analyzed in three independent experiments, except for mutants that appeared in the 70-100% category in two independent experiments. |
impaired mutants (those with pulldownload ratio <20% of positive control; see Table 1) yielded a signal intensity in the p115-RH precipitate that exceeded 50% of intensity for the positive control myc-αG12ΔQ (data not shown).

The αG12 cassette mutant designated OO was among those impaired in LARG binding, consistent with our previous work demonstrating its uncoupling from Rho-mediated signaling [31], and several other cassette substitutions within the Switch regions disrupted binding to LARG (mutants HH, LL, MM, NN, QQ, and RR; see Figure 2). However, impaired LARG binding also was caused by substitutions in other regions of αG12 (Table 1). Prior crystallographic studies identified several residues in αG13 that serve as contact points with p115-RH [20,21]. Table 2 lists αG13 residues identified as contact points with p115-RH in these earlier studies, and indicates the corresponding αG12 cassette mutant for each αG13 residue. From our in vitro binding results (Table 1), it is apparent that most αG12 mutants corresponding to RhoGEF-contacting αG13 residues displayed partial or severe impairment of LARG binding, mutants V, BB and DDD being exceptions. However, several RhoGEF-uncoupling substitutions in αG12 (cassette mutants E, I, J, K, M, Z, NN, OO, VV, AAA, EEE, FFF, GGG and HHH) replaced amino acids that do not correspond to αG13 contacts with p115-RH. αG12 mutants J and K replaced sections of the P-loop, a motif critical in guanine nucleotide binding, and thus would be predicted as impaired in signaling. However, our finding of RhoGEF-uncoupling

Table 2 αG12 cassette mutants corresponding to rgRGS contact points within αG13

| αG13 residues in contact with p115-RH | αG12 cassette mutants |
|-------------------------------------|----------------------|
| Val-208                             | Q                    |
| Asp-101, Ala-102                     | R                    |
| Lys-105, Leu-106                     | S                    |
| Thr-127, Arg-128                     | V                    |
| Phe-168                             | BB                   |
| Arg-200, Pro-202, Lys-204            | HH                   |
| Gln-226                             | LL                   |
| Arg-230, Lys-231, Phe-234            | MM                   |
| Met-257                             | QQ                   |
| Arg-260                             | RR                   |
| Asn-270                             | SS                   |
| Ile-271, Glu-273, Thr-274, Ile-275   | TT                   |
| Asn-278, Arg-279, Val-280            | UU                   |
| Arg-335                             | DDD                  |

αG12 native residues previously identified as providing contact points with the RH domain of p115RhoGEF [20,21] are indicated in the left column. Cassette mutants (“NAAIRS” substitution) in which the homologous residue(s) within αG12 have been altered are indicated in the right column. See Figure 2 for αG12 mutant designations.

mutations at the N- and C-termini of αG12 was unexpected, because these regions either lacked corresponding contact points in the αG13/p115-RH complex or were disordered in the G12/13 crystal structures (i.e. the N-terminus). To determine whether these N- and C-terminal mutations in αG12 are impaired in Rho-mediated signaling, we expressed these variants in HEK293 cells and measured stimulation of SRE-luciferase transcription. All N- and C-terminal mutants impaired in RhoGEF binding were poor activators of this reporter gene (Figure 4A). Several cassette mutants in the N- and C-terminal regions of αG12 that displayed normal binding to LARG (mutants E, V, and KKK) stimulated SRE-luciferase in a manner comparable to the myc-αG12ΔQ positive control (Figure 4A). With the exception of mutant VV, immunoblot analysis of HEK293 cell lysates revealed expression levels of these mutants similar to myc-αG12ΔQ (Figure 4B).

Conformational activation of RhoGEF-uncoupled αG12 mutants

A concern in our experimental approach was that specific “NAAIRS” cassette substitutions could cause global disruption of αG12 shape, so that a mutant might fail to assume an activated conformation. For RhoGEF-uncoupled αG12 mutants at the N-terminus (i.e. upstream of the P-loop) and C-terminus, we measured protection against trypsin proteolysis. Exchange of GDP for the activating GTP on Gα proteins triggers a conformational change that conceals a trypsin cleavage site within the Switch II region; this property allows the activated state of the Gα protein to be revealed by resistance to trypsin [25,32]. As shown in Figure 5A, mutants E, I, and HHH yielded a protected fragment of approximately 40 kDa that matched the fragment observed following tryptic digestion of myc-αG12ΔQ. Results for mutant AAA were difficult to interpret; a band of slightly smaller size than undigested AAA was generated by trypsin digestion, but it was unclear whether this matched the ~40 kDa trypsin-protected fragment in myc-αG12ΔQ. Other C-terminal mutants we tested– VV, EEE, FFF, and GGG– appeared to match the constitutively inactive myc-αG12ΔQ E228A which lacked this ~40 kDa fragment (Figure 5A). These results suggest several C-terminal mutants of αG12 were sufficiently distorted in shape by the “NAAIRS” substitution to allow trypsin access to proteolytic sites normally not exposed in the GTP-bound state. However, cassette mutants E and I at the N-terminus and HHH at the C-terminus appeared to maintain an activated conformation despite their impairment in RhoGEF binding and SRE stimulation.

We also tested whether RhoGEF-uncoupled cassette mutants at the N- and C-termini of αG12 could interact in vitro with other reported binding partners: heat shock protein-90, protein phosphatase-5, the scaffolding Aα
Figure 4 Activation of serum response element mediated transcription by Go12 mutants. (A) Luciferase reporter assay results of selected cassette mutants. HEK293 cells grown in 12-well plates were co-transfected with the plasmids SRE-L (0.2 μg) and pRL-TK (0.02 μg), plus 1.0 μg of the plasmid encoding each cassette mutant indicated on the X-axis. Firefly luciferase values were normalized for Renilla luciferase values within each sample, and values are presented as a percent of the value calculated for myc-Gα12QL (Y-axis) within the same experiment. Mutationally active (12QL) and inactive (G228A) samples were analyzed in parallel. Results shown are a representative of two experiments performed per Gα12 variant. (B) Expression level of Gα12 mutants. A sample of each lysate was set aside prior to luminometry and analyzed by SDS-PAGE and immunoblotting using anti-Gα12 antibody (Santa Cruz Biotechnology). For all samples, densitometric intensity was determined as described in Methods, then divided by positive control myc-Gα12QL levels within the same experiment, and SRE-L/Renilla values were adjusted to reflect this normalization for protein levels.

Figure 5 Conformational efficacy of N-terminal and C-terminal Gα12 mutants uncoupled from RhoGEFs. (A) Trypsin protection of selected Gα12 mutants. HEK293 cell lysates expressing the indicated variants of myc-Gα12QL, or unmodified myc-Gα12QL (12QL), or the G228A variant of myc-Gα12 (12G228A) were subjected to trypsin protection assays as described in Methods. Samples were incubated 20 min at 30°C in the presence (+) or absence (−) of TPCK-treated trypsin, and were analyzed by SDS-PAGE and immunoblotting using J169 antibody (1:700 dilution). Small horizontal arrows indicate position of the trypsin-protected fragment in selected lanes. Data presented are representative of two or more independent experiments per sample. (B) Specificity of uncoupling in selected Gα12 variants. For each cassette mutant of myc-Gα12QL (indicated at top), interaction with each Gα12 target (indicated at left) was quantified as a pulldown:load ratio as described in Methods, and was calculated as a percent of the identical ratio determined for myc-Gα12QL within the same experiment. Values are indicated as follows: (+++) = >60%, (+) = 20 to 60%, (+) = 0 to 20%. Interacting proteins are GST fusions of the following: RH domain of LARG (LARG), C-terminal 107 amino acids of heat shock protein-90 alpha (Hsp90), protein phosphatase-5 (PP5), scaffolding Aa subunit of protein phosphatase-2A (PP2A), C-terminal 98 amino acids of E-cadherin (E-cad). Values presented indicate the mean of two or more trials per interaction sample.
subunit of protein phosphatase-2A, and the cytoplasmic tail of E-cadherin [33-36]. As shown in Figure 5B, each mutant displayed pulldown:load ratios >60% of the positive control, myc-Gq12QL, for at least two of these non-RhoGEF targets. Taken as a whole, these findings reveal a subset of mutations at the N- and C-terminus that selectively uncouple Gq12 from RhoGEFs while preserving conformational activation and ability to bind other downstream proteins.

We next sought to identify specific residues within these N- and C-terminal sextets of Gq12 that mediate RhoGEF interaction. To examine putative surface residues, we performed charge substitutions in the native regions corresponding to cassette mutants E, I, and HHH, and examined these variants for SRE-luciferase activation. None of the single-residue charge-reversals in the regions encompassed in mutants I or HHH caused significant decrease in SRE signaling (data not shown). However, a double charge-reversal in the mutant E region, converting Glu31 and Glu33 to Arg residues, caused a near-complete loss of SRE activation in HEK293 cells despite normal levels of protein expression (Figure 7A). We next examined this Gq12 mutant, designated Glu31/33Arg, for binding to the RH domains of LARG and p115RhoGEF. As shown in Figure 7B, a selective loss of RhoGEF binding was observed: the Glu31/33Arg charge-reversals severely disrupted LARG-RH binding relative to non-mutated myc-Gq12QL (pulldown:load ratio ~18% of control) but had minimal effect on p115-RH binding (ratio ~86% of control). In trypsin protection assays, the Glu31/33Arg mutant yielded a protected fragment at the same molecular weight (~40 kDa) as observed for the myc-Gq12QL positive control, suggesting its ability to attain an activated conformation (Figure 7C). The intermediate intensity of this band (approximately a midpoint between activated Gq12 and the constitutively inactive Gly228Ala variant) may be due in part to the mutational introduction of Arg residues providing additional sites for trypsin proteolysis. Taken as a whole, these findings not only provide evidence that the structurally uncharacterized N-terminus of Gq12 plays a role in its functional interaction with RhoGEFs, but also reveal individual charged residues in this region as candidates for conferring specificity of Gq12 for LARG among the RH-containing RhoGEFs.

**Figure 6 Structural position of Gα12 C-terminal determinants of RhoGEF binding.** The structure of N-terminally Gα12-substituted Gα12 (PDB accession code 1ZCA, [22]) as a GDP-αIF2-activated complex was analyzed using PyMOL software. The native Gα12 region substituted for the sequence “NAAIRS” in the C-terminal mutants EEF, FFF, and GGG is highlighted in orange, and the sextet substituted in mutant HHH is highlighted in black. The bound GDP molecule is highlighted in blue. Figure was rendered in The PyMOL Molecular Graphics System, Version 1.5.0.1 Schrödinger, LLC.

**Discussion**

The G12 subfamily members Gα12 and Gα13 are well-documented as utilizing RhoGEFs as downstream signaling effectors. Crystallographic studies by Chen et al. [20] and Hajicek et al. [21] have provided intricate structural details of the interaction between Gα13 and the RH domain of p115RhoGEF, identifying a set of Gα13 residues that directly contact this target protein. The structure of Gα12 also has been elucidated, using a chimera comprised of amino acids 49–379 of Gα12 preceded by amino acids 1–28 of Gα13 [22]. However, a Gα12:RhoGEF complex has not been reported. In the current study, we utilized in vitro and cell-based approaches to examine the interaction between Gα12 and two putative target RhoGEFs, LARG and p115RhoGEF. Using immobilized RGS-homology (RH) domains of these RhoGEFs, we identified several substitutions of native amino acids in Gα12 that disrupted its binding to these proteins and blocked its ability to stimulate the Rho-dependent process of SRE-mediated transcription. Although our results indicated that a number of common determinants
in Go12 and Go13 mediate RhoGEF binding, several RhoGEF-uncoupling mutations in Go12 did not correspond to regions of RhoGEF contact within Go13; these include amino acid sextet substitutions in the C-terminal α5 helix as well as the structurally uncharacterized N-terminus . Several of these Go12 mutants exhibited protection from tryptic digestion as well as unimpeded binding to other, non-RhoGEF targets, indicating their impaired interaction with RhoGEFs is not caused by failure to attain an activated conformation and suggesting the shapes of other effector-binding surfaces in these Go12 mutants remain intact as RhoGEF interaction is disrupted.

Although Go12 and Go13 share 67% amino acid identity and bind several common downstream targets, several functional differences between these Gα proteins suggest their signaling mechanisms are not redundant [1,3]. Both Go12 and Go13 bind LARG and p115RhoGEF [10,12], and both of these RhoGEFs accelerate GTPase activity of purified Go12 and Go13 in single-turnover assays [13,17]. Whereas Go13 stimulates both p115RhoGEF and LARG to trigger guanine nucleotide exchange on RhoA in vitro, Go12 can only stimulate LARG under these experimental conditions, and in a manner dependent on prior phosphorylation of LARG by the tyrosine kinase Tec [10,13]. Also, activated Go12 is more potent than Go13 in recruiting the RH domain of p115RhoGEF to the plasma membrane, and specific mutations in p115RhoGEF disrupt Go12 but not Go13 in triggering this localization [37]. At the cellular and organismal levels, it is increasingly clear that Go12 and Go13 utilize non-overlapping signaling pathways. Mice lacking Go13 die early in embryogenesis due to defects in vascular development and thrombin-induced cell migration, but mice lacking Go12 do not display these developmental defects. However, knockout of Go12 combined with absence of Go13 causes earlier lethality than Go13 knockout alone, and in mice lacking one Go13 allele, at least one Go12 allele must be present for normal embryonic development [38,39]. Furthermore, LPA-induced activation of mTOR complex 2 leading to activation of PKC-δ requires Go12 but not Go13 [40]. Because of these differences, plus the increasing list of Go12-specific effector proteins (including another RhoGEF, AKAP-Lbc, that is activated exclusively by Go12 within the G12/13 subfamily), we believe the Go12-RhoGEF interface cannot be defined summarily by structural features of the Go13-RhoGEF complex.

Among the Go13 residues that provide contact points with p115RhoGEF in crystallographic studies [20,21], many have corresponding residues within Go12, and therefore we paid particular attention to Go12 cassette mutants corresponding to these key Go13 residues (see Table 2). For example, the Go12 mutant HH replaced residues corresponding to Go13 residues Arg200, and
Lys\(^{204}\), both of which provide contact points with p115-RH. In another G\(_{\alpha_{12}}\) cassette mutant, termed RR, a substituted residue corresponds to Arg\(^{260}\) within G\(_{\alpha_{13}}\); this residue provides a key contact with amino acids within the βN-αN region of p115RhoGEF. Also, G\(_{\alpha_{12}}\) cassette mutants Q, R, and S contain altered residues in the G\(_{\alpha_{12}}\) helical domain that correspond to p115-RH interacting residues in G\(_{\alpha_{13}}\). Among the G\(_{\alpha_{12}}\) mutants corresponding to p115-RH contact points in G\(_{\alpha_{13}}\), most showed impaired RhoGEF interaction and poor stimulation of SRE-mediated signaling. However, several differences between G\(_{\alpha_{12}}\) and G\(_{\alpha_{13}}\) were noted, particularly in the helical domain. G\(_{\alpha_{12}}\) cassette mutant V alters residues that correspond to two contact points within the G\(_{\alpha_{13}}\)p115-RH complex; however, this mutant showed minimal impairment in RhoGEF binding in vitro and stimulated SRE-mediated transcription robustly in cells. G\(_{\alpha_{12}}\) mutant BB, which removes a Phe corresponding to a G\(_{\alpha_{13}}\) contact point with p115-RH, displayed a slight impairment in SRE-mediated transcriptional activation and no impairment of RhoGEF binding. In addition, G\(_{\alpha_{13}}\) utilizes a C-terminal residue (Arg\(^{335}\)) as a contact point with p115-RH, but the corresponding G\(_{\alpha_{12}}\) cassette mutant (DDD) exhibited normal binding to RhoGEFs and only modest impairment in SRE signaling. However, because this cassette mutant preserves the corresponding Arg residue in G\(_{\alpha_{12}}\) (DRKRRN substituted for NAAIRS), it is possible this Arg in G\(_{\alpha_{12}}\) participates in RhoGEF binding despite the alteration in adjacent amino acids.

Aside from the N- and C-terminal mutants of G\(_{\alpha_{12}}\) that show impaired RhoGEF binding, we have identified other RhoGEF-uncoupling mutations in G\(_{\alpha_{12}}\) that lack corresponding G\(_{\alpha_{13}}\) contact points for p115-RH (see Tables 1 and 2). None of the native G\(_{\alpha_{12}}\) residues replaced in cassette mutants M and Z match p115-RH contact points in G\(_{\alpha_{13}}\), and thus may indicate G\(_{\alpha_{12}}\)-specific determinants of RhoGEF interaction. Impaired RhoGEF binding also was observed in G\(_{\alpha_{12}}\) mutants J and K; however, this was likely due to these substitutions disrupting the canonical GXXGXXGKS guanine nucleotide binding motif [41]. Although our results suggest a core similarity in the mechanisms utilized by G\(_{\alpha_{12}}\) and G\(_{\alpha_{13}}\) to engage RhoGEF targets, it is apparent that several determinants of RhoGEF binding are unique to G\(_{\alpha_{13}}\). We have identified determinants that may be unique to G\(_{\alpha_{12}}\) or potentially important for both G12/13 subfamily members in RhoGEF engagement. Studies of G\(_{\alpha_{13}}\) variants harbor corresponding mutations will be important in distinguishing these possibilities.

A role for the C-terminus of G12/13 subfamily proteins in RhoGEF engagement has been suggested by prior studies. Kreutz et al. [42] engineered chimeras of G\(_{\alpha_{12}}\) and G\(_{\alpha_{13}}\) that were interchanged downstream of the Switch III region, and demonstrated the C-terminal 114 amino acids of G\(_{\alpha_{13}}\) as sufficient for its unique ability to stimulate purified p115RhoGEF to activate RhoA. Also, a chimeric G\(_{\alpha_{13}}\) in which the region downstream of Switch III was replaced by the corresponding region of G\(_{\alpha_{12}}\) displayed loss of ability to stimulate SRE-mediated transcriptional activation [43]. Initial crystallographic studies of G\(_{\alpha_{13}}\)-RhoGEF interaction utilized a chimeric G\(_{\alpha_{13}}\) harboring G\(_{\alpha_{12}}\) sequence at the C-terminus, and determinants of RhoGEF binding were not found downstream of the Switch regions in this protein [20]. Subsequent crystallographic work utilizing G\(_{\alpha_{13}}\) with native C-terminal sequence did identify residues slightly downstream of the Switch III region as critical for RhoGEF engagement [21], and also revealed a more distal residue in the C-terminal region (Arg\(^{385}\)) as providing a contact point with the RH domain of p115RhoGEF. However, no residues at the extreme C-terminus of G\(_{\alpha_{13}}\), including the α5 helix, were found to mediate RhoGEF binding. Our results suggest differences between G\(_{\alpha_{12}}\) and G\(_{\alpha_{13}}\) in the role of the C-terminus, as several substitutions near the extreme C-terminus of G\(_{\alpha_{12}}\) disrupted RhoGEF interaction, most notably the cassette mutant HHH within the α5 helix.

The N-terminus provides the greatest amino acid sequence divergence between G\(_{\alpha_{12}}\) and G\(_{\alpha_{13}}\). Ga subunits utilize this region for interaction with Gβγ [44], and in G\(_{\alpha_{12}}\) and G\(_{\alpha_{13}}\) this region confers specificity of coupling to thrombin and LPA receptors, respectively [19]. Importantly, G\(_{\alpha_{13}}\) is a more potent stimulator of RhoGEF activation in vitro than a chimeric G\(_{\alpha_{13}}\) harboring the N-terminus of G\(_{\alpha_{12}}\), indicating a possible role of the G\(_{\alpha_{13}}\) N-terminus in RhoGEF activation [21]. However, specific determinants within the N-terminus of G12/13 subfamily proteins that mediate binding to effectors, including RhoGEFs, have not been reported. The 48-residue region at the N-terminus of G\(_{\alpha_{12}}\) has not been characterized in crystallographic studies, because its replacement by the G\(_{\alpha_{1}}\) N-terminus was necessary for obtaining sufficient quantities of purified protein [16,22]. Furthermore, the N-terminus was disordered in crystallographic analysis of both the aforementioned G\(_{\alpha_{1}}\)/G\(_{\alpha_{13}}\) hybrid and a more recent structure of full-length G\(_{\alpha_{13}}\) [21], suggesting the G\(_{\alpha_{12}}\) N-terminus may be refractory to crystallographic analysis even if native sequence is utilized. Our approach of employing cassette substitution mutants throughout the length of G\(_{\alpha_{12}}\) has provided an indirect means of circumventing this obstacle, and has revealed specific N-terminal regions as possible determinants of RhoGEF interaction. Importantly, our discovery that mutations in this N-terminal region (cassette mutants E and J) cause loss of RhoGEF binding allowed us to focus on putative surface residues in these substituted regions, ultimately revealing Glu\(^{31}\)
and Glu\textsuperscript{33} as critical for G\textsubscript{\alpha\textsubscript{12}} interaction with LARG and stimulation of SRE-mediated transcription. Our finding that charge substitutions of these N-terminal G\textsubscript{\alpha\textsubscript{12}} residues disrupted binding to the LARG-RH domain but had minimal effect on interaction with the corresponding domain of p115RhoGEF was intriguing, and suggested these residues play a role in targeting G\textsubscript{\alpha\textsubscript{12}} preferentially to LARG. It is possible that G\textsubscript{\alpha\textsubscript{12}} harbors sufficient RhoGEF-interacting surfaces for \textit{in vitro} binding to p115RhoGEF, but that a functional, physiological interaction (i.e. with LARG) requires this N-terminal region. Our RhoGEF binding results for G\textsubscript{\alpha\textsubscript{12}} cassette mutant E, as well as the more specific Glu\textsuperscript{31/33}Arg mutant, were surprising in light of earlier findings that RhoGEF binding was preserved in a G\textsubscript{\alpha\textsubscript{12}} chimera containing the Ga\textsubscript{i} N-terminus [22]. It is possible that “NAAIRS” substitution and particularly the Glu\textsuperscript{31/33}Arg charge-reversals cause a more dramatic change to this RhoGEF binding surface than occurs when Ga\textsubscript{i} sequence is introduced. Cassette mutant E and the Glu\textsuperscript{31/33}Arg mutant are impaired in activating the Rho-dependent readout of SRE-mediated transcriptional activation in cells, and it remains to be determined whether the Ga\textsubscript{i}/G\textsubscript{\alpha\textsubscript{12}} chimera is similarly impaired in stimulating this pathway.

Because previous phosphorylation of LARG by Tec is a requirement for G\textsubscript{\alpha\textsubscript{12}}, but not Ga\textsubscript{i}, for \textit{in vitro} activation of Rho, it will be important to determine whether this phosphorylation event regulates interaction of LARG with G\textsubscript{\alpha\textsubscript{12}}, particularly its N-terminus and C-terminal \alpha\textsubscript{5} helix. Furthermore, as suggested by Hajicek et al. [21], it is conceivable that post-translational modification of p115RhoGEF in cells modulates its responsiveness to G\textsubscript{\alpha\textsubscript{i1}} or could potentially render it a target of G\textsubscript{\alpha\textsubscript{12}}. A challenge for future studies of G\textsubscript{\alpha\textsubscript{12}}- and Ga\textsubscript{i1}-mediated signaling will be to determine the combinations of G\textsubscript{\alpha\textsubscript{12}} and Ga\textsubscript{i1} subunits and RhoGEFs that activate Rho in response to different signaling inputs, and in different cell and tissue types.

Conclusions

G\textsubscript{\alpha\textsubscript{12}} and Ga\textsubscript{i1} define the G12/13 class of heterotrimeric G protein \alpha-subunits, which participate in numerous signaling pathways through stimulation of RhoGEFs that subsequently activate Rho. Although these proteins are non-redundant in their stimulation of effectors and their cellular and organismal roles, only Ga\textsubscript{i1} has been characterized in the structural basis of its interaction with RhoGEF targets. However, the involvement of G\textsubscript{\alpha\textsubscript{12}} in stimulating SRE-mediated transcription, cell rounding, c-Jun N-terminal kinase activation, cell growth, and metastatic invasion supports a physiological role for a G\textsubscript{\alpha\textsubscript{12}}-RhoGEF-Rho axis in developmental pathways and disease progression [45]. Therefore, an improved understanding of the structural aspects of G\textsubscript{\alpha\textsubscript{12}}-RhoGEF interaction likely will be of broad importance. Our results provide several key additions to this structural model: 1) characterization of the G\textsubscript{\alpha\textsubscript{12}}:RhoGEF interacting surface by identifying regions in G\textsubscript{\alpha\textsubscript{12}} that mediate binding; 2) unexpected roles of the G\textsubscript{\alpha\textsubscript{12}} N-terminal region and C-terminal \alpha\textsubscript{5} helix in engagement of RhoGEFs; 3) identification of specific residues near the G\textsubscript{\alpha\textsubscript{12}} N-terminus that may mediate its selectivity for LARG as an effector protein. To date, no structural studies have examined the interaction of G\textsubscript{\alpha\textsubscript{12}} with RhoGEFs. Our hope is that mutant-based strategies will augment such crystallographic approaches and provide key details toward understanding the structural aspects and biological role of this G\textsubscript{\alpha}:effector interaction.

Methods

DNA constructs

Plasmids encoding 1) a fusion of glutathione-S-transferase (GST) to amino acids 320–606 of LARG (GST-LARG-RH), and 2) amino acids 1–252 of p115RhoGEF with an N-terminal myc epitope tag were kindly provided by Tohru Kozasa (Univ. of Ill., Chicago). We used PCR to subclone the p115RhoGEF sequence into pGEX-2T (GE Healthcare) to produce GST-p115-RH. All “NAAIRS” amino acid substitution mutants within myc-tagged G\textsubscript{\alpha\textsubscript{12}} Gln\textsuperscript{229}Leu (myc-G\textsubscript{\alpha\textsubscript{12}}\textsuperscript{QL}) were engineered as described previously [29]. Single amino acid substitutions were engineered in myc-G\textsubscript{\alpha\textsubscript{12}}\textsuperscript{QL} using the QuikChange II\textsuperscript{®} site-directed mutagenesis system (Agilent Technologies), and this system was used to engineer a constitutively inactive Gly\textsuperscript{228}Ala variant (myc-G\textsubscript{\alpha\textsubscript{12}}\textsuperscript{QL,G228A}) within a plasmid encoding myc-tagged, wildtype G\textsubscript{\alpha\textsubscript{12}} (provided by Pat Casey, Duke University). The luciferase reporter plasmid SRE-L was a gift from Channing Der (University of North Carolina Chapel Hill).

Expression and immobilization of GST fusion proteins

GST fusion constructs were transformed into BL21 (Gold)-DE3 cells (Stratagene). Cells were grown under 75 \mu g/ml ampicillin selection to OD\textsubscript{600} of 0.5–0.7, and recombinant protein expression was induced using 0.5 mM isopropyl-\beta-D-thiogalactopyranoside (Fisher Scientific). After 3 h, cells were lysed on ice using 0.32 mg/ml lysozyme (MP Biomedicals), and GST fusion proteins were bound to glutathione-sepharose 4B (GE Healthcare) as described previously [31,34]. Following three washes in 50 mM Tris pH 7.7 supplemented with 1 mM EDTA, 1 mM dithiothreitol, and 150 mM NaCl, samples were snap-frozen in aliquots and stored at \textminus80°C.

Preparation of detergent-soluble extracts harboring G\textsubscript{\alpha\textsubscript{12}} mutants

Human embryonic kidney cells (HEK293) were grown in Dulbecco’s modified Eagle medium (Mediatech, Manassas,
VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin and streptomycin. For myc-Gα\(_{12}\)\(^{QL}\) and each of its 62 NAAIRS substitution mutants (see Figure 2), 7.0 μg of plasmid DNA was transfected into a 10-cm dish of HEK293 cells grown to approximate 90% confluence, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After 36–42 hours, cells were scraped from dishes, washed twice with phosphate-buffered saline, and solubilized in NAAIRS Lysis buffer (50 mM HEPES pH 7.5, 1 mM EDTA, 3 mM dithiothreitol, 10 mM MgSO\(_4\), 1% (w/v) polyoxyethylene-10-lauryl ether) containing the protease inhibitors 4–(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (1.67 mM), leupeptin (2.1 μM), pepstatin (1.45 μM), TLCK (58 μM), TPCK (61 μM), and phenylmethylsulfonyl fluoride (267 μM). Samples were centrifuged at 80,000 g for 1 h, and supernatants were snap-frozen in 60-μl aliquots and stored at −80°C.

**Protein interaction assays**

HEK293 cell extracts were diluted in NAAIRS Lysis buffer lacking polyoxyethylene-10-lauryl ether, using sufficient volume to dilute this detergent in the samples to 0.05% (w/v). Next, sepharose-bound GST fusion proteins were added and allowed to incubate for approximately 2 h at 4°C with continuous inversion. A percentage of the diluted extract was set aside as starting material prior to sepharose addition. Next, samples were centrifuged at 1,300 g, and pellets were washed three times and then subjected to SDS-PAGE and immunoblot analysis using an antibody specific to the Gα\(_{12}\) N-terminus (Santa Cruz Biotechnology) or the myc 9E10 epitope tag (Zymed), followed by alkaline phosphatase conjugated secondary antibodies (Promega). For each variant of myc-Gα\(_{12}\)\(^{QL}\), the Gaussian intensity of the ~45 kDa band from the precipitated band and the corresponding band from the starting material were quantified using a Kodak Gel Logic 100 system equipped with Molecular Imaging 5.X software (Carestream Health, New Haven CT).

**Reporter gene assays**

HEK293 cells grown in 12-well plates were transfected with 0.2 μg SRE-luciferase plasmid (encoding firefly luciferase) and 0.02 μg pRL-TK plasmid encoding Renilla luciferase, plus plasmids encoding variants of myc-Gα\(_{12}\)\(^{QL}\). Reporter assays for SRE-mediated transcriptional activation were performed as described previously [31]. Briefly, cells were washed with phosphate-buffered saline and lysed in 1X passive lysis buffer (Promega), and lysates were analyzed using a Dual-luciferase assay system and GloMax 20/20 luminometer (Promega). Light output due to firefly luciferase activity was divided by output from Renilla luciferase activity to normalize samples for transfection efficiency.

**Trypsin protection experiments**

HEK293 cells grown in 10-cm dishes were transfected with various Gα\(_{12}\) constructs using Lipofectamine 2000 (Invitrogen), and tryptic digestions were performed as a modification of the procedure of Kozasa and Gilman [25]. Briefly, cells were lysed in 50 mM Hepes pH 8.0, 1 mM EDTA, 3 mM dithiothreitol, 1% polyoxyethylene-10-lauryl ether containing the same protease inhibitors as NAAIRS Lysis buffer (see above) but at two-fold lower concentration. Samples were cleared by centrifugation at 70,000 g for 1 h, and supernatants were diluted 20-fold in volume using 50 mM Hepes pH 8.0, 1 mM EDTA, 3 mM dithiothreitol, 10 mM MgSO\(_4\). Samples were digested with 10 μg/ml TPCK-treated trypsin (New England Biolabs) for 20 min at 30°C, and proteolysis was terminated by addition of 100 μg/ml lima bean trypsin inhibitor (Worthington, Lakewood NJ). Samples were analyzed by SDS-PAGE and immunoblotting using J169 antisera specific to the Gα\(_{12}\) C-terminus, provided by Tohru Kozasa (Univ. of Ill., Chicago).

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**Authors’ information**

TEM is an affiliate member of the UNC Lineberger Comprehensive Cancer Center (Chapel Hill, NC).

**Abbreviations**

Gα\(_{12}\) and Gα\(_{13}\): Heterotrimeric guanine nucleotide binding protein α-subunits of the G12/13 subfamily; GST: Glutathione-S-transferase; HEK: Human embryonic kidney; LARG: Leukemia-associated RhoGEF; LPA: Lysophosphatidic acid; NAAIRS mutant: Variant of Gα\(_{12}\) in which a consecutive sextet of native residues has been replaced by Asn-Ala-Ala-Ile-Arg-Ser; RGS: Regulator of G protein signaling; RT: RGS homology; RhoGEF: Rho-specific guanine nucleotide exchange factor; SRE: Serum response element.

**Competing interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

BJR and WCS participated in design of the study, carried out PCR-based mutagenesis, designed and executed protein interaction screens, and participated in drafting the manuscript. In addition, BJR performed reporter assays and WCS performed 3-D protein imaging and analysis. ERM, ESF, TYC, and CMO engineered various GST-fusion constructs and carried out protein interaction screens. LAF participated in initial design of the study and carried out pilot experiments. TEM conceived of the study, participated in its design, coordination, engineering of constructs and data collection, and drafted the manuscript. All authors have read and approved the final manuscript.

**Authors**

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**Contributions**

BJR and WCS participated in design of the study, carried out PCR-based mutagenesis, designed and executed protein interaction screens, and participated in drafting the manuscript. In addition, BJR performed reporter assays and WCS performed 3-D protein imaging and analysis. ERM, ESF, TYC, and CMO engineered various GST-fusion constructs and carried out protein interaction screens. LAF participated in initial design of the study and carried out pilot experiments. TEM conceived of the study, participated in its design, coordination, engineering of constructs and data collection, and drafted the manuscript. All authors have read and approved the final manuscript.
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