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Determinants at the N- and C-termini of $G\alpha_{12}$ required for activation of Rho-mediated signaling

Benjamin J Ritchie†, William C Smolski†, Ellyn R Montgomery, Elizabeth S Fisher, Tina Y Choi, Calla M Olson, Lori A Foster and Thomas E Meigs*

Abstract

Background: Heterotrimeric guanine nucleotide binding proteins of the G12/13 subfamily, which includes the α-subunits $G\alpha_{12}$ and $G\alpha_{13}$, 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 $G\alpha_{13}$ and RhoGEFs have been examined in crystallographic studies of the purified complex, whereas a $G\alpha_{12}$:RhoGEF complex has not been reported. Several signaling responses and effector interactions appear unique to $G\alpha_{12}$ or $G\alpha_{13}$, despite their similarity in amino acid sequence.

Methods: To comprehensively examine $G\alpha_{12}$ for regions involved in RhoGEF interaction, we screened a panel of $G\alpha_{12}$ 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 $G\alpha_{12}$ binding to LARG and the related p115RhoGEF. These $G\alpha_{12}$ mutants also were impaired in activating serum response element mediated signaling, a Rho-dependent response. Most of these mutants matched corresponding regions of $G\alpha_{13}$ reported to contact p115RhoGEF, but unexpectedly, several RhoGEF-uncoupling mutations were found within the N- and C-terminal regions of $G\alpha_{12}$. Trypsin protection assays revealed several mutants in these regions as retaining conformational activation. In addition, charge substitutions near the $G\alpha_{12}$ N-terminus selectively disrupted binding to LARG but not p115RhoGEF.

Conclusions: Several structural aspects of the $G\alpha_{12}$:RhoGEF interface differ from the reported $G\alpha_{13}$:RhoGEF complex, particularly determinants within the C-terminal α_5 helix and structurally uncharacterized N-terminus of $G\alpha_{12}$. Furthermore, key residues at the $G\alpha_{12}$ N-terminus may confer selectivity for LARG as a downstream effector.

Keywords: $G\alpha_{12}$, $G\alpha_{13}$, 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, $G\alpha_{12}$ and $G\alpha_{13}$, 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 Gα-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 $G\alpha_{12}$ to trigger increased invasiveness of breast cancer cells [9].

* Correspondence: tmeigs@unca.edu
†Equal contributors
Department of Biology, University of North Carolina at Asheville, One University Heights, Asheville, NC 28804, USA

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 $G\alpha_{12}$ and $G\alpha_{13}$ and accelerates GTPase activity for both proteins, but only $G\alpha_{13}$ can stimulate p115RhoGEF to activate RhoA *in vitro* [10,17]. Interaction of $G\alpha_{12}$ or $G\alpha_{13}$ with purified LARG can trigger its activation of RhoA; however, stimulation by $G\alpha_{12}$ 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 $G\alpha_{12}$, whereas the LPA receptor preferentially utilizes $G\alpha_{13}$ as a conduit to downstream signaling [19]. Although it is possible that $G\alpha_{12}$ 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 $G\alpha_{12}$. Gains in our understanding of the specificity of RhoGEF engagement within the G12/13 subfamily should provide insights into the non-overlapping functions of $G\alpha_{12}$ and $G\alpha_{13}$ in signal transduction.

Crystallographic studies have revealed important structural aspects of the interaction between $G\alpha_{13}$ and the RH domain of p115RhoGEF, including numerous residues in both proteins that provide contact points [20,21]. Initially, purification of $G\alpha_{13}$ 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 $G\alpha i_1$ [20]. The structure of the $G\alpha_{13}$: p115RhoGEF-RH complex was later refined in crystallographic studies that utilized a $G\alpha_{13}$ chimera harboring $G\alpha i_1$ sequence only at the N-terminus. Because the Gα N-terminus was unstructured in this crystallized complex, any role of this region in RhoGEF interaction remains to be determined. Although the region of $G\alpha_{13}$ downstream of the Switch III region harbors several residues critical for RhoGEF engagement, notably Glu^{273}, Thr^{274}, Asn^{278}, and Arg^{279} within the α_3 helix and α_3-β_5 loop, other regions closer to the $G\alpha_{13}$ C-terminus do not emerge in the crystal structure as providing key RhoGEF contact points [21].

In contrast to $G\alpha_{13}$, a structure of $G\alpha_{12}$ in complex with a RhoGEF target has not been reported, although a chimeric $G\alpha_{12}$ harboring the N-terminus of $G\alpha i_1$ has been crystallized [22]. To examine the full sequence of $G\alpha_{12}$ for structural features mediating its interaction with RhoGEFs, we engineered a series of cassette substitutions within constitutively activated $G\alpha_{12}$ 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 $G\alpha_{12}$ as harboring determinants of its functional interaction with RhoGEFs, and also identify key charged amino acids near the $G\alpha_{12}$ N-terminus that may confer selective binding to LARG.

Results

Myc-tagged $G\alpha_{12}$ retains RhoGEF binding, Rho-mediated signaling, and conformational activation

To identify mutants of $G\alpha_{12}$ impaired in RhoGEF binding, we first sought to establish an *in vitro* system in which $G\alpha_{12}$ mutants could be expressed ectopically in cultured cells, rendered soluble in a detergent extract, and detected without interference from endogenous $G\alpha_{12}$. We engineered the constitutively active $Gln^{229}Leu$ variant of $G\alpha_{12}$ ($G\alpha_{12}^{QL}$) to harbor a myc epitope tag, flanked by linkers of the sequence SGGGGS and positioned between residues Pro^{139} and Val^{140}. This insertion site was chosen due to its approximate alignment with the position of green fluorescent protein in Gαq in a prior study [24]. We expressed myc-tagged and untagged $G\alpha_{12}^{QL}$ in HEK293 cells, prepared detergent-soluble extracts, and analyzed these by immunoblotting. As shown in Figure 1A, myc-tagged $G\alpha_{12}^{QL}$ was detected by both anti-myc and anti-$G\alpha_{12}$ 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 $G\alpha_{12}$ and untagged $G\alpha_{12}^{QL}$ (~43 kDa). Next, we subjected myc-$G\alpha_{12}^{QL}$ to pulldown experiments using an immobilized GST fusion of the p115RhoGEF RH domain, as described in Methods. Myc-tagged and untagged $G\alpha_{12}^{QL}$ bound to p115-RH with similar affinity (Figure 1B), and comparison with mock-transfected cells indicated the ~45 kDa band detected by anti-$G\alpha_{12}$ was dependent on transfection with the myc-$G\alpha_{12}^{QL}$ plasmid. Furthermore, LARG-RH and p115-RH showed similar ability to co-precipitate myc-tagged $G\alpha_{12}^{QL}$ (Figure 1C). To ascertain that myc-$G\alpha_{12}$ 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

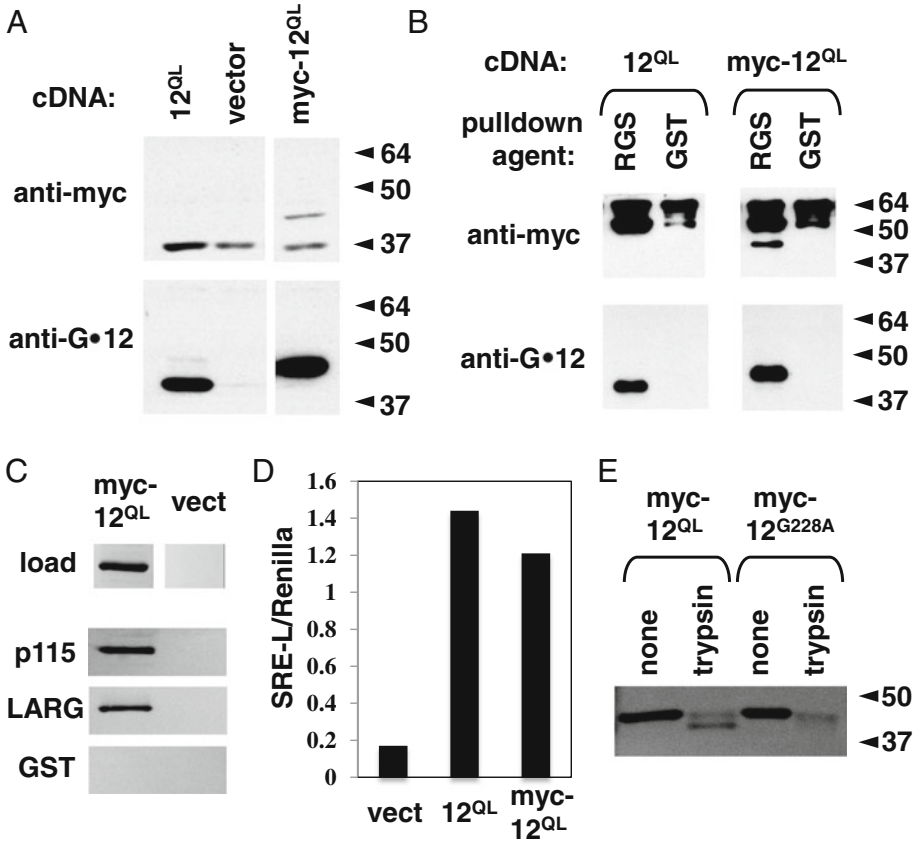


Figure 1 Effector binding and conformational activation of myc-tagged, constitutively activated $G\alpha_{12}$. Molecular weight markers (in kDa) are indicated at right of panels where applicable. All results shown are representative of two or more independent experiments. (**A**) Expression and solubilization of $G\alpha_{12}^{QL}$ (*12^{QL}*) and myc-tagged $G\alpha_{12}^{QL}$ (*myc-12^{QL}*) transiently expressed in HEK293 cells. Cells transfected with the vector pcDNA3.1 are included as a negative control (*vector*). Detergent-soluble extracts were prepared by high-speed centrifugation and subjected to SDS-PAGE and immunoblotting, using either anti-myc (Zymed) or anti-$G\alpha_{12}$ (Santa Cruz Biotechnology) antibodies as described in Methods. (**B**) *In vitro* binding of myc-tagged and untagged $G\alpha_{12}^{QL}$ by p115RhoGEF. HEK293 cells extracts containing myc-$G\alpha_{12}^{QL}$ were subjected to protein interaction assays (see Methods) using an immobilized GST fusion of the RH domain of p115RhoGEF (*RGS*) or GST alone (*GST*). Samples were washed, separated by SDS-PAGE, and analyzed by immunoblotting using antibodies described above. (**C**) Specificity of myc-$G\alpha_{12}^{QL}$ detection in interaction assays. HEK293 cells transfected with either myc-$G\alpha_{12}^{QL}$ (*myc-12^{QL}*) or the empty pcDNA3.1 plasmid (*vect*) were lysed and assayed for binding to GST fusions of the RH domain of p115RhoGEF (*p115*) or LARG, or GST alone (*GST*). Immunoblot analysis was performed using anti-$G\alpha_{12}$ antibody as described above. (**D**) Serum response element (SRE) luciferase activation by myc-$G\alpha_{12}^{QL}$. 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 0.1 μg of the plasmid indicated on the X-axis. Y-axis values show firefly luciferase signal normalized for *Renilla* luciferase signal within each sample. (**E**) Trypsin protection assays of myc-tagged $G\alpha_{12}$. Lysates from HEK293 cells transfected with myc-$G\alpha_{12}^{QL}$ (*myc-12^{QL}*) or the constitutively GDP-bound $Gly^{228}Ala$ mutant of wildtype $G\alpha_{12}$ (*myc-12^{G228A}*) were subjected to trypsin digests as described in Methods. Immunoblot analysis was performed using J169 antibody [25] at 1:700 dilution.

element (SRE), a component of the c-fos promoter that provides a readout of $G\alpha_{12}$-mediated Rho activation [23]. Myc-tagged and untagged $G\alpha_{12}^{QL}$ 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-$G\alpha_{12}^{QL}$ yielded a protected fragment of ~40 kDa, comparable to results observed previously with GTPγS-loaded, purified $G\alpha_{12}$ [25]. An inactive, constitutively GDP-bound ($Gly^{228}Ala$) variant of myc-tagged $G\alpha_{12}$ did not yield this ~40 kDa fragment when digested with trypsin (Figure 1E). Taken together, these results suggest myc-$G\alpha_{12}^{QL}$ undergoes conformational activation and retains normal signaling through the RhoGEF:Rho pathway. Because of the superior sensitivity of anti-$G\alpha_{12}$ antibody in detecting myc-$G\alpha_{12}^{QL}$, and the easily discernible gel shift of $G\alpha_{12}$ caused by the myc tag and linkers (see Figures 1A and B), we chose to utilize anti-$G\alpha_{12}$ to detect myc-$G\alpha_{12}^{QL}$ in subsequent protein binding experiments.

Mutations that uncouple $G\alpha_{12}$ from RhoGEF binding and Rho-mediated signaling

To scan $G\alpha_{12}$ for regions participating in its interaction with RhoGEFs, we utilized a comprehensive panel of

mutants in which sextets of consecutive amino acids in myc-$G\alpha_{12}^{QL}$ 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 $G\alpha_{12}$ determinants of binding to the scaffolding subunit of protein phosphatase-2A and the cytoplasmic tail of polycystin-1 [29,30]. Variants of $G\alpha_{12}$ were expressed in HEK293 cells and tested for interaction with immobilized LARG-RH, as described in Methods. As shown in Figure 3, myc-$G\alpha_{12}^{QL}$ 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-$G\alpha_{12}^{QL}$ 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-$G\alpha_{12}^{QL}$ 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

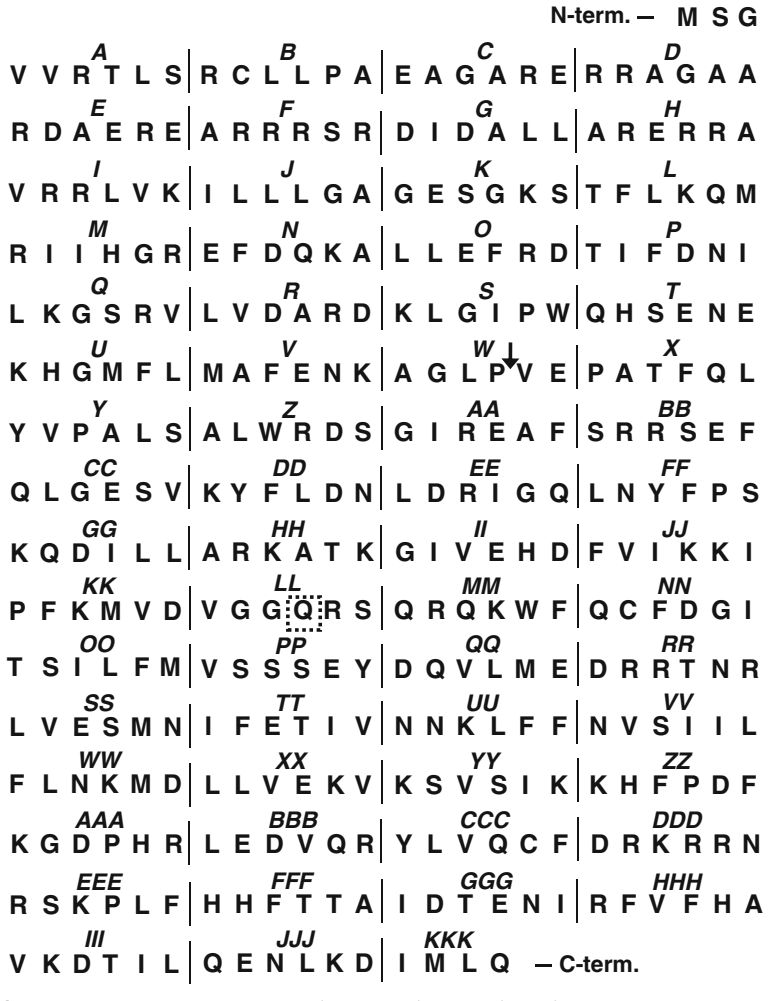


Figure 2 Residues replaced in $G\alpha_{12}$ cassette mutants. For each mutant, designated in italics (*A-Z*, *AA-ZZ*, *AAA-KKK*), the native amino acid sextet replaced by the sequence Asn-Ala-Ala-Ile-Arg-Ser is shown. An arrow between Pro^{139} and Val^{140} indicates the site of myc tag insertion. Mutant *W* was not produced. The dashed box indicates the native Gln^{229} mutated to Leu to render $G\alpha_{12}$ constitutively active. The native residues replaced in mutant *KKK* are Lys-Asp-Ile-Met-Leu-Gln and thus partially overlap with mutant *JJJ*. All cassette mutants contain the activating $Q^{229}L$ mutation, except mutant *LL* due to its cassette substitution.

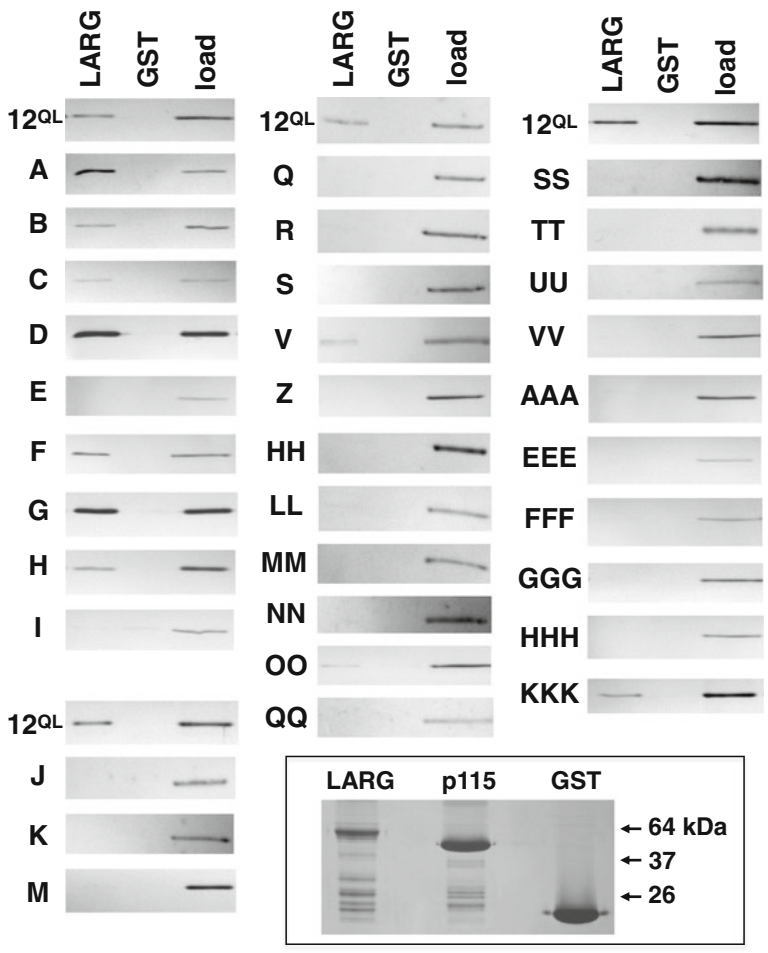


Figure 3 In vitro interaction of $G\alpha_{12}$ mutants with LARG. Immunoblot results for all LARG binding-impaired $G\alpha_{12}$ cassette mutants and selected other mutants are shown. HEK293 cells were transfected with the indicated plasmids (7.0 μg per 10-cm plate) and lysates were prepared for co-precipitation assays as described in Methods. Prior to this step, 5% of each lysate was set aside as starting material (*load*). Pulldown experiments were performed on 7–9 mutants per experiment, plus myc-$G\alpha_{12}^{QL}$ 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.

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-$G\alpha_{12}^{QL}$. However, a number of mutants exhibited lower pulldown:load ratios (<20% of positive control) with a subset generating a ratio less than 10% of the positive control. For all samples, precipitation by immobilized GST yielded no $G\alpha_{12}$ signal (Figure 3), indicating these mutants were not merely binding the GST-glutathione-sepharose complex nor forming insoluble aggregates under these *in vitro* conditions. Also, we examined the full panel of $G\alpha_{12}$ cassette mutants for interaction with a GST fusion of the N-terminal 252 amino acids of p115RhoGEF (p115-RH). None of the LARG binding-

Table 1 $G\alpha_{12}$ cassette mutants impaired in binding LARG-RH

70-100%	A-D F-H L N O T-V X BB DD-FF II KK XX YY BBB DDD III-KKK
40-70%	H P GG JJ WW ZZ CCC
20-40%	Y AA PP
10-20%	I OO QQ-SS UU VV FFF
0-10%	E J K M Q R S Z HH LL-NN TT AAA EEE GGG HHH
N/D	W CC

Cassette substitution mutants of myc-$G\alpha_{12}^{QL}$ (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-$G\alpha_{12}^{QL}$ assayed in parallel. Each $G\alpha_{12}$ 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 pulldown:load 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-$G\alpha_{12}^{QL}$ (data not shown).

The $G\alpha_{12}$ 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 $G\alpha_{12}$ (Table 1). Prior crystallographic studies identified several residues in $G\alpha_{13}$ that serve as contact points with p115-RH [20,21]. Table 2 lists $G\alpha_{13}$ residues identified as contact points with p115-RH in these earlier studies, and indicates the corresponding $G\alpha_{12}$ cassette mutant for each $G\alpha_{13}$ residue. From our *in vitro* binding results (Table 1), it is apparent that most $G\alpha_{12}$ mutants corresponding to RhoGEF-contacting $G\alpha_{13}$ residues displayed partial or severe impairment of LARG binding, mutants *V, BB* and *DDD* being exceptions. However, several RhoGEF-uncoupling substitutions in $G\alpha_{12}$ (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 $G\alpha_{13}$ contacts with p115-RH. $G\alpha_{12}$ 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 mutations at the N- and C-termini of $G\alpha_{12}$ was unexpected, because these regions either lacked corresponding contact points in the $G\alpha_{13}$: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 $G\alpha_{12}$ 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 $G\alpha_{12}$ that displayed normal binding to LARG (mutants *F, V,* and *KKK*) stimulated SRE-luciferase in a manner comparable to the myc-$G\alpha_{12}^{QL}$ 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-$G\alpha_{12}^{QL}$ (Figure 4B).

Table 2 $G\alpha_{12}$ cassette mutants corresponding to rgRGS contact points within $G\alpha_{13}$

$G\alpha_{13}$ residues in contact with p115-RH	myc-$G\alpha_{12}^{QL}$ NAAIRS mutant
Val^{98}	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

$G\alpha_{13}$-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 $G\alpha_{12}$ have been altered are indicated in the right column. See Figure 2 for $G\alpha_{12}$ mutant designations.

Conformational activation of RhoGEF-uncoupled $G\alpha_{12}$ mutants

A concern in our experimental approach was that specific "NAAIRS" cassette substitutions could cause global disruption of $G\alpha_{12}$ shape, so that a mutant might fail to assume an activated conformation. For RhoGEF-uncoupled $G\alpha_{12}$ 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-$G\alpha_{12}^{QL}$. Results for mutant *AAA* were difficult to interpret; a band of slightly smaller size than undigested *AAA* was generated by tryptic digestion, but it was unclear whether this matched the ~40 kDa trypsin-protected fragment in myc-$G\alpha_{12}^{QL}$. Other C-terminal mutants we tested– *VV, EEE*, *FFF*, and *GGG*– appeared to match the constitutively inactive myc-$G\alpha_{12}^{G228A}$ which lacked this ~40 kDa fragment (Figure 5A). These results suggest several C-terminal mutants of $G\alpha_{12}$ 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 $G\alpha_{12}$ could interact *in vitro* with other reported binding partners: heat shock protein-90, protein phosphatase-5, the scaffolding Aα

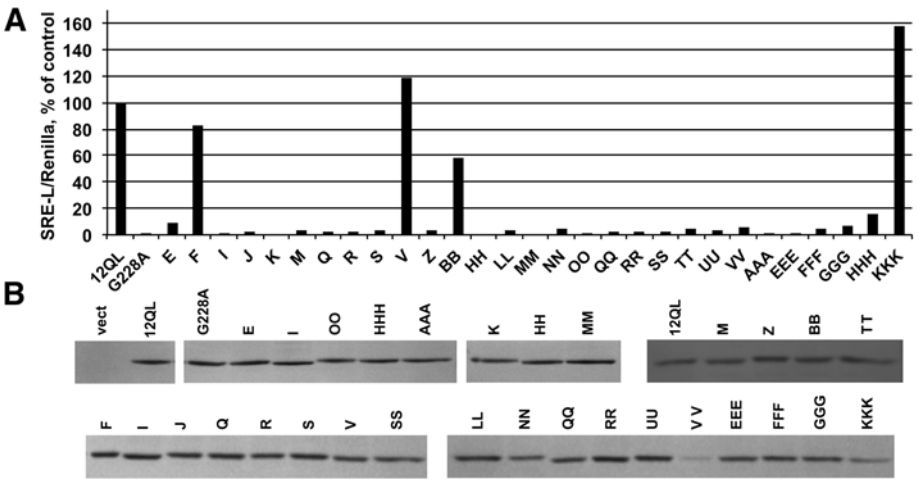
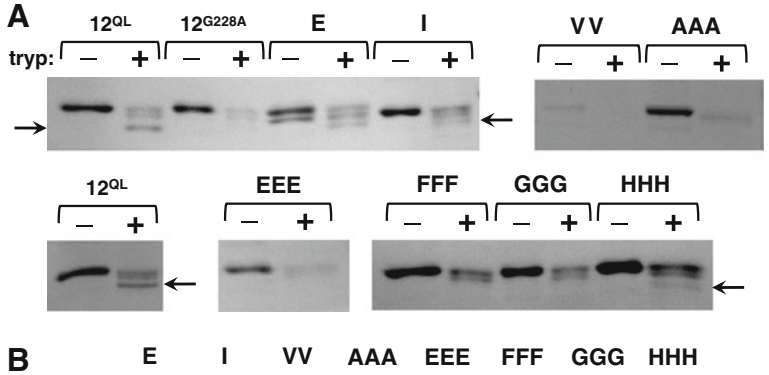


Figure 4 Activation of serum response element mediated transcription by Gα12 mutants. (**A**) Luciferase reporter assay results of selected cassette mutants. HEK293 cells grown in 12-well plates were co-transfected with the plasmids SRE-L (0.2 μg) and pRL-TK (0.02 μg), plus 1.0 μg of the plasmid encoding 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-$Gα_{12}^{QL}$ (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 $Gα_{12}$ variant. (**B**) Expression level of $Gα_{12}$ mutants. A sample of each lysate was set aside prior to luminometry and analyzed by SDS-PAGE and immunoblotting using anti-$Gα_{12}$ antibody (Santa Cruz Biotechnology). For all samples, densitometric intensity was determined as described in Methods, then divided by positive control myc-$Gα_{12}^{QL}$ levels within the same experiment, and SRE-L/*Renilla* values were adjusted to reflect this normalization for protein levels.



B

	E	I	VV	AAA	EEE	FFF	GGG	HHH
LARG	–	–	–	–	–	–	–	–
Hsp90	–/+	+	++	–/+	+/++	++	+	++
PP5	++	++	+/++	++	++	++	++	++
PP2A	++	–	++	–	+	+	++	–
E-cad	–	++	+	+	++	++	++	++

Figure 5 Conformational efficacy of N-terminal and C-terminal $Gα_{12}$ mutants uncoupled from RhoGEFs. (**A**) Trypsin protection of selected $Gα_{12}$ mutants. HEK293 cell lysates expressing the indicated variants of myc-$Gα_{12}^{QL}$, or unmodified myc-$Gα_{12}^{QL}$ (12^{QL}), or the G^{228}A variant of myc-$Gα_{12}$ (12^{G228A}) 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 $Gα_{12}$ variants. For each cassette mutant of myc-$Gα_{12}^{QL}$ (indicated at top), interaction with each $Gα_{12}$ 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-$Gα_{12}^{QL}$ 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 Aα 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-$G\alpha_{12}^{QL}$, 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 $G\alpha_{12}$ from RhoGEFs while preserving conformational activation and ability to bind other downstream proteins.

We next visualized the position of these RhoGEF-interacting regions in the crystal structure of a $G\alpha_{12}$ chimera in which the N-terminal 48 residues were replaced by the N-terminus of $G\alpha i_1$ [22]. The native region of $G\alpha_{12}$ replaced in cassette mutant *E* is not ordered in this structure; however, the regions replaced in the C-terminal mutants *EEE-HHH* are highlighted (Figure 6). The sextet replaced in mutant *HHH* (highlighted in black) resides in the α_5 helix that extends along the $G\alpha_{12}$ surface and approaches the C-terminus at the top of the diagram.

Differential uncoupling of $G\alpha_{12}$ from LARG and p115RhoGEF

We next sought to identify specific residues within these N- and C-terminal sextets of $G\alpha_{12}$ 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 Glu^{31} and Glu^{33} 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 $G\alpha_{12}$ mutant, designated $Glu^{31/33}$Arg, for binding to the RH domains of LARG and p115RhoGEF. As shown in Figure 7B, a selective loss of RhoGEF binding was observed: the $Glu^{31/33}$Arg charge-reversals severely disrupted LARG-RH binding relative to non-mutated myc-$G\alpha_{12}^{QL}$ (pulldown:load ratio ~18% of control) but had minimal effect on p115-RH binding (ratio ~86% of control). In trypsin protection assays, the $Glu^{31/33}$Arg mutant yielded a protected fragment at the same molecular weight (~40 kDa) as observed for the myc-$G\alpha_{12}^{QL}$ positive control, suggesting its ability to attain an activated conformation (Figure 7C). The intermediate intensity of this band (approximately a midpoint between activated $G\alpha_{12}$ and the constitutively inactive Gly^{228}Ala 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 $G\alpha_{12}$ plays a role in its functional interaction with RhoGEFs, but also reveal individual charged residues in this region as candidates for conferring specificity of $G\alpha_{12}$ for LARG among the RH-containing RhoGEFs.

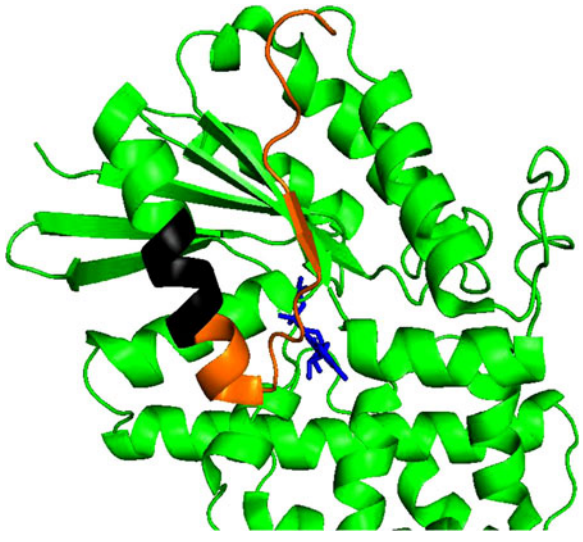


Figure 6 Structural position of $G\alpha_{12}$ C-terminal determinants of RhoGEF binding. The structure of N-terminally $G\alpha i_1$-substituted $G\alpha_{12}$ (PDB accession code 1ZCA, [22]) as a GDP•AlF_4^- activated complex was analyzed using PyMOL software. The native $G\alpha_{12}$ region substituted for the sequence "NAAIRS" in the C-terminal mutants *EEE*, *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 $G\alpha_{12}$ and $G\alpha_{13}$ 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 $G\alpha_{13}$ and the RH domain of p115RhoGEF, identifying a set of $G\alpha_{13}$ residues that directly contact this target protein. The structure of $G\alpha_{12}$ also has been elucidated, using a chimera comprised of amino acids 49–379 of $G\alpha_{12}$ preceded by amino acids 1–28 of $G\alpha i_1$ [22]. However, a $G\alpha_{12}$:RhoGEF complex has not been reported. In the current study, we utilized *in vitro* and cell-based approaches to examine the interaction between $G\alpha_{12}$ 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 $G\alpha_{12}$ 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

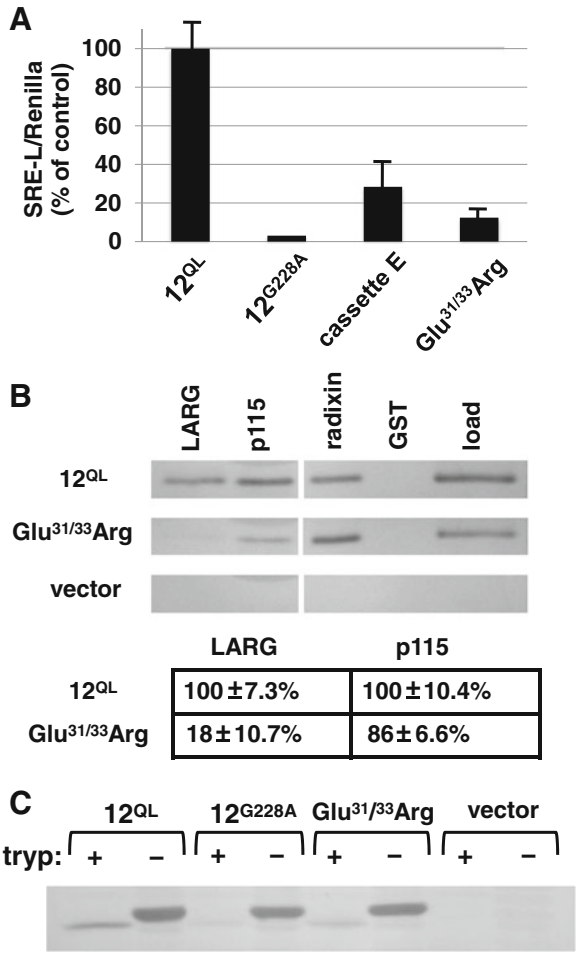


Figure 7 Selective RhoGEF uncoupling by N-terminal charge substitutions in $G\alpha_{12}$. (**A**) Luciferase reporter gene assays. Cassette mutant *E* (see Figure 2) and the double charge substitution mutant $Glu^{31/33}Arg$ were compared to myc-$G\alpha_{12}^{QL}$ (12^{QL}) in SRE-luciferase assays under the cell transfection conditions described in Figure 4. A constitutively GDP-bound variant of wildtype myc-$G\alpha_{12}$ (12^{G228A}) was assayed in parallel as a negative control. Results shown are the mean of three independent experiments, and error bars indicate range. (**B**) Protein-protein interaction assays. Detergent-soluble extracts from transfected HEK293 cells transfected with myc-$G\alpha_{12}^{QL}$, the $Glu^{31/33}Arg$ mutant, or empty pcDNA3.1 plasmid (*vector*) were subjected to co-precipitation assays as described in Methods, using GST-fusions of either LARG-RH (*LARG*), p115RhoGEF-RH (*p115*), the N-terminal domain of the $G\alpha_{12}$ target radixin [46], or no adduct (*GST*). Prior to the precipitation step, 5% of each lysate was set aside as starting material (*load*). Table values show the pulldown:load ratio for $Glu^{31/33}Arg$ as a percent of the positive control value (12^{QL}), with mean +/- range presented for three independent experiments. (**C**) Trypsin protection of the $Glu^{31/33}Arg$ mutant, in comparison to constitutively GTP- and GDP-bound $G\alpha_{12}$. Assays were performed as described in Methods. Results shown are representative of two independent experiments.

in $G\alpha_{12}$ and $G\alpha_{13}$ mediate RhoGEF binding, several RhoGEF-uncoupling mutations in $G\alpha_{12}$ did not correspond to regions of RhoGEF contact within $G\alpha_{13}$; these include amino acid sextet substitutions in the C-terminal α_5 helix as well as the structurally uncharacterized N-terminus . Several of these $G\alpha_{12}$ 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 $G\alpha_{12}$ mutants remain intact as RhoGEF interaction is disrupted.

Although $G\alpha_{12}$ and $G\alpha_{13}$ 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 $G\alpha_{12}$ and $G\alpha_{13}$ bind LARG and p115RhoGEF [10,12], and both of these RhoGEFs accelerate GTPase activity of purified $G\alpha_{12}$ and $G\alpha_{13}$ in single-turnover assays [13,17]. Whereas $G\alpha_{13}$ stimulates both p115RhoGEF and LARG to trigger guanine nucleotide exchange on RhoA *in vitro*, $G\alpha_{12}$ 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 $G\alpha_{12}$ is more potent than $G\alpha_{13}$ in recruiting the RH domain of p115RhoGEF to the plasma membrane, and specific mutations in p115RhoGEF disrupt $G\alpha_{12}$ but not $G\alpha_{13}$ in triggering this localization [37]. At the cellular and organismal levels, it is increasingly clear that $G\alpha_{12}$ and $G\alpha_{13}$ utilize non-overlapping signaling pathways. Mice lacking $G\alpha_{13}$ die early in embryogenesis due to defects in vascular development and thrombin-induced cell migration, but mice lacking $G\alpha_{12}$ do not display these developmental defects. However, knockout of $G\alpha_{12}$ combined with absence of $G\alpha_{13}$ causes earlier lethality than $G\alpha_{13}$ knockout alone, and in mice lacking one $G\alpha_{13}$ allele, at least one $G\alpha_{12}$ 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 $G\alpha_{12}$ but not $G\alpha_{13}$ [40]. Because of these differences, plus the increasing list of $G\alpha_{12}$-specific effector proteins (including another RhoGEF, AKAP-Lbc, that is activated exclusively by $G\alpha_{12}$ within the G12/13 subfamily), we believe the $G\alpha_{12}$:RhoGEF interface cannot be defined summarily by structural features of the $G\alpha_{13}$:RhoGEF complex.

Among the $G\alpha_{13}$ residues that provide contact points with p115RhoGEF in crystallographic studies [20,21], many have corresponding residues within $G\alpha_{12}$, and therefore we paid particular attention to $G\alpha_{12}$ cassette mutants corresponding to these key $G\alpha_{13}$ residues (see Table 2). For example, the $G\alpha_{12}$ mutant *HH* replaced residues corresponding to $G\alpha_{13}$ residues Arg^{200}, 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 most likely was due to these substitutions disrupting the canonical GXGXXGKS 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 harboring 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 $G\alpha i_2$ 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 $G\alpha i_1$ 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^{335}) 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 α_5 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 α_5 helix.

The N-terminus provides the greatest amino acid sequence divergence between $G\alpha_{12}$ and $G\alpha_{13}$. Gα 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 $G\alpha i_1$, 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 $G\alpha i_1$ 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 $G\alpha i_1/G\alpha_{13}$ 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 *I*) cause loss of RhoGEF binding allowed us to focus on putative surface residues in these substituted regions, ultimately revealing Glu^{31}

and Glu^{33} as critical for $G\alpha_{12}$ interaction with LARG and stimulation of SRE-mediated transcription. Our finding that charge substitutions of these N-terminal $G\alpha_{12}$ 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 $G\alpha_{12}$ preferentially to LARG. It is possible that $G\alpha_{12}$ harbors sufficient RhoGEF-interacting surfaces for *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 $G\alpha_{12}$ cassette mutant *E*, as well as the more specific $Glu^{31/33}$Arg mutant, were surprising in light of earlier findings that RhoGEF binding was preserved in a $G\alpha_{12}$ chimera containing the $G\alpha i_1$ N-terminus [22]. It is possible that "NAAIRS" substitution and particularly the $Glu^{31/33}$Arg charge-reversals cause a more dramatic change to this RhoGEF binding surface than occurs when $G\alpha i_1$ sequence is introduced. Cassette mutant *E* and the $Glu^{31/33}$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 $G\alpha i_1/G\alpha_{12}$ chimera is similarly impaired in stimulating this pathway.

Because previous phosphorylation of LARG by Tec is a requirement for $G\alpha_{12}$, but not $G\alpha_{13}$, for *in vitro* activation of Rho, it will be important to determine whether this phosphorylation event regulates interaction of LARG with $G\alpha_{12}$, particularly its N-terminus and C-terminal α_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 $G\alpha_{13}$ or could potentially render it a target of $G\alpha_{12}$. A challenge for future studies of $G\alpha_{12}$- and $G\alpha_{13}$-mediated signaling will be to determine the combinations of G12/13 subfamily α-subunits and RhoGEFs that activate Rho in response to different signaling inputs, and in different cell and tissue types.

Conclusions

$G\alpha_{12}$ and $G\alpha_{13}$ define the G12/13 class of heterotrimeric G protein α-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 $G\alpha_{13}$ has been characterized in the structural basis of its interaction with RhoGEF targets. However, the involvement of $G\alpha_{12}$ in stimulating SRE-mediated transcription, cell rounding, c-Jun N-terminal kinase activation, cell growth, and metastatic invasion supports a physiological role for a $G\alpha_{12}$-RhoGEF-Rho axis in developmental pathways and disease progression [45]. Therefore, an improved understanding of the structural aspects of $G\alpha_{12}$:RhoGEF interaction likely will be of broad importance. Our results provide several key additions to this structural model: 1) characterization of the $G\alpha_{12}$:RhoGEF interacting surface by identifying regions in $G\alpha_{12}$ that mediate binding; 2) unexpected roles of the $G\alpha_{12}$ N-terminal region and C-terminal α_5 helix in engagement of RhoGEFs; 3) identification of specific residues near the $G\alpha_{12}$ N-terminus that may mediate its selectivity for LARG as an effector protein. To date, no structural studies have examined the interaction of $G\alpha_{12}$ 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 Gα: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 $G\alpha_{12}$ Gln^{229}Leu (myc-$G\alpha_{12}^{QL}$) were engineered as described previously [29]. Single amino acid substitutions were engineered in myc-$G\alpha_{12}^{QL}$ using the QuikChange II® site-directed mutagenesis system (Agilent Technologies), and this system was used to engineer a constitutively inactive Gly^{228}Ala variant (myc-$G\alpha_{12}^{G228A}$) within a plasmid encoding myc-tagged, wildtype $G\alpha_{12}$ (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 μg/ml ampicillin selection to OD_{600} of 0.5–0.7, and recombinant protein expression was induced using 0.5 mM isopropyl-β-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 −80°C.

Preparation of detergent-soluble extracts harboring $G\alpha_{12}$ 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-$G\alpha_{12}^{QL}$ 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 $G\alpha_{12}$ 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-$G\alpha_{12}^{QL}$, the Gaussian intensity of the ~45 kDa band from the precipitated material 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-$G\alpha_{12}^{QL}$. 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 $G\alpha_{12}$ 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 $G\alpha_{12}$ C-terminus, provided by Tohru Kozasa (Univ. of Ill., Chicago).

Abbreviations

$G\alpha_{12}$ and $G\alpha_{13}$: 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 $G\alpha_{12}$ in which a consecutive sextet of native residues has been replaced by Asn-Ala-Ala-Ile-Arg-Ser; RGS: Regulator of G protein signaling; RH: 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' information

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

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References

1. Worzfeld T, Wettschureck N, Offermanns S: **G_{12}/G_{13}-mediated signalling in mammalian physiology and disease.** *Trends Pharmacol Sci* 2008, **29:**582–589.
2. Suzuki N, Hajicek N, Kozasa T: **Regulation and physiological functions of G12/13-mediated signaling pathways.** *Neurosignals* 2009, **17:**55–70.
3. Kelly P, Casey PJ, Meigs TE: **Biologic functions of the G12 subfamily of heterotrimeric G proteins: growth, migration, and metastasis.** *Biochemistry* 2007, **46:**6677–6687.
4. Sternweis PC, Carter AM, Chen Z, Danesh SM, Hsiung YF, Singer WD: **Regulation of Rho guanine nucleotide exchange factors by G proteins.** *Adv Protein Chem* 2007, **74:**189–228.
5. Jaffe AB, Hall A: **Rho GTPases: biochemistry and biology.** *Annu Rev Cell Dev Biol* 2005, **21:**247–269.
6. Barrett K, Leptin M, Settleman J: **The Rho GTPase and a putative RhoGEF mediate a signaling pathway for the cell shape changes in Drosophila gastrulation.** *Cell* 1997, **91:**905–915.
7. Yau DM, Yokoyama N, Goshima Y, Siddiqui ZK, Siddiqui SS, Kozasa T: **Identification and molecular characterization of the Gα12-Rho guanine nucleotide exchange factor pathway in Caenorhabditis elegans.** *Proc Natl Acad Sci USA* 2003, **100:**14748–14753.
8. Lin F, Sepich DS, Chen S, Topczewski J, Yin C, Solnica-Krezel L, Hamm H: **Essential roles of $G\alpha_{12/13}$ signaling in distinct cell behaviors driving zebrafish convergence and extension gastrulation movements.** *J Cell Biol* 2005, **169:**777–787.
9. Kelly P, Moeller BJ, Juneja J, Booden MA, Der CJ, Daaka Y, Dewhirst MW, Fields TA, Casey PJ: **The G12 family of heterotrimeric G proteins promotes breast cancer invasion and metastasis.** *Proc Natl Acad Sci USA* 2006, **103:**8173–8178.
10. Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG, Sternweis PC, Bollag G: **Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by $G\alpha_{13}$.** *Science* 1998, **280:**2112–2114.
11. Fukuhara S, Murga C, Zohar M, Igishi T, Gutkind JS: **A novel PDZ domain containing guanine nucleotide exchange factor links heterotrimeric G proteins to Rho.** *J Biol Chem* 1999, **274:**5868–5879.
12. Fukuhara S, Chikumi H, Gutkind JS: **Leukemia-associated Rho guanine nucleotide exchange factor (LARG) links heterotrimeric G proteins of the G(12) family to Rho.** *FEBS Lett* 2000, **485:**183–188.
13. Suzuki N, Nakamura S, Mano H, Kozasa T: **Gα12 activates Rho GTPase through tyrosine-phosphorylated leukemia-associated RhoGEF.** *Proc Natl Acad Sci USA* 2003, **100:**733–738.
14. Hollinger S, Hepler JR: **Cellular regulation of RGS proteins: modulators and integrators of G protein signaling.** *Pharmacol Rev* 2002, **54:**527–559.
15. Siderovski DP, Willard FS: **The GAPs, GEFs, and GDIs of heterotrimeric G-protein alpha subunits.** *Int J Biol Sci* 2005, **1:**51–66.
16. Chen Z, Singer WD, Danesh SM, Sternweis PC, Sprang SR: **Recognition of the activated states of Gα13 by the rgRGS domain of PDZRhoGEF.** *Structure* 2008, **16:**1532–1543.
17. Kozasa T, Jiang X, Hart MJ, Sternweis PM, Singer WD, Gilman AG, Bollag G, Sternweis PC: **p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$.** *Science* 1998, **280:**2109–2111.
18. Wang Q, Liu M, Kozasa T, Rothstein JD, Sternweis PC, Neubig RR: **Thrombin and lysophosphatidic acid receptors utilize distinct rhoGEFs in prostate cancer cells.** *J Biol Chem* 2004, **279:**28831–28834.
19. Yamaguchi Y, Katoh H, Negishi M: **N-terminal short sequences of α subunits of the G_{12} family determine selective coupling to receptors.** *J Biol Chem* 2003, **278:**14936–14939.
20. Chen Z, Singer WD, Sternweis PC, Sprang SR: **Structure of the p115RhoGEF rgRGS domain-Gα13/i1 chimera complex suggests convergent evolution of a GTPase activator.** *Nat Struct Mol Biol* 2005, **12:**191–197.
21. Hajicek N, Kukimoto-Niino M, Mishima-Tsumagari C, Chow CR, Shirouzu M, Terada T, Patel M, Yokoyama S, Kozasa T: **Identification of critical residues in $G\alpha_{13}$ for stimulation of p115RhoGEF activity and the structure of the $G\alpha_{13}$-p115RhoGEF regulator of G protein signaling homology (RH) domain complex.** *J Biol Chem* 2011, **286:**20625–20636.
22. Kreutz B, Yau DM, Nance MR, Tanabe S, Tesmer JJG, Kozasa T: **A new approach to producing functional Gα subunits yields the activated and deactivated structures of $G\alpha_{12/13}$ proteins.** *Biochemistry* 2006, **45:**167–174.
23. Fromm C, Coso OA, Montaner S, Xu N, Gutkind JS: **The small GTP-binding protein Rho links G protein-coupled receptors and $G\alpha_{12}$ to the serum response element and to cellular transformation.** *Proc Natl Acad Sci USA* 1997, **94:**10098–10103.
24. Hughes TE, Zhang H, Logothetis DE, Berlot CH: **Visualization of a functional Gαq-green fluorescent protein fusion in living cells. Association with the plasma membrane is disrupted by mutational activation and by elimination of palmitoylation sites, but not by activation mediated by receptors or AlF_4^-.** *J Biol Chem* 2001, **276:**4227–4235.
25. Kozasa T, Gilman AG: **Purification of recombinant G proteins from Sf9 cells by hexahistidine tagging of associated subunits. Characterization of α_{12} and inhibition of adenylyl cyclase by α_z.** *J Biol Chem* 1995, **270:**1734–1741.
26. Wilson IA, Haft DH, Getzoff ED, Tainer JA, Lerner RA, Brenner S: **Identical short peptide sequences in unrelated proteins can have different conformations: a testing ground for theories of immune recognition.** *Proc Natl Acad Sci USA* 1985, **82:**5255–5259.
27. Sellers WR, Novitch BG, Miyake S, Heith A, Otterson GA, Kaye FJ, Lassar AB, Kaelin WG: **Stable binding to E2F is not required for the retinoblastoma protein to activate transcription, promote differentiation, and suppress tumor cell growth.** *Genes Dev* 1998, **12:**95–106.
28. Armbruster BN, Banik SS, Guo C, Smith AC, Counter CM: **N-terminal domains of the human telomerase catalytic subunit required for enzyme activity in vivo.** *Mol Cell Biol* 2001, **21:**7775–7786.
29. Zhu D, Tate RI, Ruediger R, Meigs TE, Denker BM: **Domains necessary for $G\alpha_{12}$ binding and stimulation of protein phosphatase-2A (PP2A): Is $G\alpha_{12}$ a novel regulatory subunit of PP2A?** *Mol Pharmacol* 2007, **71:**1268–1276.
30. Yu W, Ritchie BJ, Su X, Zhou J, Meigs TE, Denker BM: **Identification of polycystin-1 and Gα12 binding regions necessary for regulation of apoptosis.** *Cell Signal* 2011, **23:**213–221.
31. Meigs TE, Juneja J, DeMarco CT, Stemmle LN, Kaplan DD, Casey PJ: **Selective uncoupling of $G\alpha_{12}$ from Rho-mediated signaling.** *J Biol Chem* 2005, **280:**18049–18055.
32. Miller RT, Masters SB, Sullivan KA, Beiderman B, Bourne HR: **A mutation that prevents GTP-dependent activation of the α chain of G_s.** *Nature* 1988, **334:**712–715.
33. Vaiskunaite R, Kozasa T, Voyno-Yasenetskaya TA: **Interaction between the Gα subunit of heterotrimeric G_{12} protein and Hsp90 is required for $G\alpha_{12}$ signaling.** *J Biol Chem* 2001, **276:**46088–46093.
34. Meigs TE, Fields TA, McKee DD, Casey PJ: **Interaction of Gα12 and Gα13 with the cytoplasmic domain of cadherin provides a mechanism for β-catenin release.** *Proc Natl Acad Sci USA* 2001, **98:**519–524.
35. Yamaguchi Y, Katoh H, Mori K, Negishi M: **$G\alpha_{12}$ and $G\alpha_{13}$ interact with Ser/Thr protein phosphatase type 5 and stimulate its phosphatase activity.** *Curr Biol* 2002, **12:**1353–1358.
36. Zhu D, Kosik KS, Meigs TE, Yanamadala V, Denker BM: **$G\alpha_{12}$ directly interacts with PP2A: evidence for $G\alpha_{12}$-stimulated PP2A phosphatase activity and dephosphorylation of microtubule-associated protein, Tau.** *J Biol Chem* 2004, **279:**54983–54986.
37. Bhattacharyya R, Banerjee J, Khalili K, Wedegaertner PB: **Differences in $G\alpha_{12}$- and $G\alpha_{13}$-mediated plasma membrane recruitment of p115-RhoGEF.** *Cell Signal* 2009, **21:**996–1006.
38. Offermanns S, Mancino V, Revel JP, Simon MI: **Vascular system defects and impaired cell chemokinesis as a result of $G\alpha_{13}$ deficiency.** *Science* 1997, **275:**533–536.
39. Gu JL, Muller S, Mancino V, Offermanns S, Simon MI: **Interaction of Gα12 with Gα13 and Gαq signaling pathways.** *Proc Natl Acad Sci USA* 2002, **99:**9352–9357.
40. Gan X, Wang J, Wang C, Sommer E, Kozasa T, Srinivasula S, Alessi D, Offermanns S, Simon MI, Wu D: **PRR5L degradation promotes mTORC2-mediated PKC-δ phosphorylation and cell migration downstream of Gα12.** *Nat Cell Biol* 2012, **14:**686–696.
41. Hamm HE, Gilchrist A: **Heterotrimeric G proteins.** *Curr Opin Cell Biol* 1996, **8:**189–196.
42. Kreutz B, Hajicek N, Yau DM, Nakamura S, Kozasa T: **Distinct regions of Gα13 participate in its regulatory interactions with RGS homology domain-containing RhoGEFs.** *Cell Signal* 2007, **19:**1681–1689.
43. Vazquez-Prado J, Miyazaki H, Castellone MD, Teramoto H, Gutkind JS: **Chimeric $G\alpha_{i2}/G\alpha_{13}$ proteins reveal the structural requirements for the**

binding and activation of the RGS-like (RGL)-containing Rho guanine nucleotide exchange factors (GEFs) by $G\alpha_{13}$. *J Biol Chem* 2004, **279:**54283–54290.

44. Denker BM, Neer EJ, Schmidt CJ: **Mutagenesis of the amino terminus of the alpha subunit of the G protein G_o. In vitro characterization of $\alpha_o\beta\gamma$ interactions.** *J Biol Chem* 1992, **267:**6272–6277.
45. Juneja J, Casey PJ: **Role of G12 proteins in oncogenesis and metastasis.** *Br J Pharmacol* 2009, **158:**32–40.
46. Vaiskunaite R, Adarichev V, Furthmayr H, Kozasa T, Gudkov A, Voyno-Yasenetskaya TA: **Conformational activation of radixin by G_{13} protein α subunit.** *J Biol Chem* 2000, **275:**26206–26212.

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