Ga_12 Structural Determinants of Hsp90 Interaction Are Necessary for Serum Response Element–Mediated Transcriptional Activation


Department of Biology, University of North Carolina at Asheville, Asheville, North Carolina (E.R.M., B.K.B., J.W.M., T.P.H., A.C.T., W.C.S., T.E.M.); Departments of Biology (K.A.P., S.L.R., A.M.J.), Biochemistry and Biophysics (B.R.S.T.), Cell Biology and Physiology (C.E.T.), and Pharmacology (A.M.J.), R. L. Juliano Structural Bioinformatics Core Facility (B.R.S.T.), and Carolina Center for Genome Sciences (S.L.R.), University of North Carolina, and the Lineberger Comprehensive Cancer Center, (S.L.R., T.E.M.), Chapel Hill, North Carolina

Received July 17, 2013, accepted January 16, 2014

ABSTRACT
The G12/13 class of heterotrimeric G proteins, comprising the α-subunits Ga12 and Ga13, regulates multiple aspects of cellular behavior, including proliferation and cytoskeletal rearrangements. Although guanine nucleotide exchange factors for the monomeric G protein Rho (RhoGEFs) are well characterized as effectors of this G protein class, a variety of other downstream targets has been reported. To identify Ga12 determinants that mediate specific protein interactions, we used a structural and evolutionary comparison between the G12/13, Gα, Gβ, and Gγ classes to identify “class-distinctive” residues in Gaα12 and Gaα13. Mutation of these residues in Gaα12 to their deduced ancestral forms revealed a subset necessary for activation of serum response element (SRE)–mediated transcription, a G12/13–stimulated pathway implicated in cell proliferative signaling. Unexpectedly, this subset of Gaα12 mutants showed impaired binding to heat-shock protein 90 (Hsp90) while retaining binding to RhoGEFs. Corresponding mutants of Gaα13 exhibited robust SRE activation, suggesting a Gaα12–specific mechanism, and inhibition of Hsp90 by geldanamycin or small interfering RNA–mediated lowering of Hsp90 levels resulted in greater downregulation of Gaα12 than Gaα13 signaling in SRE activation experiments. Furthermore, the Drosophila G12/13 homolog Concertina was unable to signal to SRE in mammalian cells, and Gaα12:Concertina chimeras revealed Gaα12–specific determinants of SRE activation within the switch regions and a C-terminal region. These findings identify Gaα12 determinants of SRE activation, implicate Gaα12:Hsp90 interaction in this signaling mechanism, and illuminate structural features that arose during evolution of Gaα12 and Gaα13 to allow bifurcated mechanisms of signaling to a common cell proliferative pathway.

Introduction
The ability to perceive and respond to information from the environment is a fundamental property of living cells. G protein–coupled receptors (GPCRs) are integral membrane proteins that evolved to detect a wide range of extracellular signals, including neurotransmitters, hormones, odors, and light. On activation, GPCRs undergo a conformational change that stimulates heterotrimeric G proteins, intracellular signaling molecules that consist of a GTP-binding α-subunit that exists in 1:1:1 stoichiometry with a β- and a γ-subunit. This heterotrimer is inactive when Gα is GDP-bound and GPCR activation causes Gα to exchange GDP for GTP, dissociate from the Gβγ heterodimer, and activate downstream effector proteins (Oldham and Hamm, 2008). Ga proteins are categorized into four classes based on amino acid sequence: Gs, Gβ, Gγ, and G12/13. Although each class signals to distinct effector proteins, Ga proteins share a common fold; thus, it is widely accepted that Ga–effector interactions are dictated by class-specific amino acids that create permissive binding surfaces (Cabrera-Vera et al., 2003). Understanding the principles that govern Ga–effector interaction is essential to deciphering the role of specific signaling pathways.

The G12/13 class of Ga proteins is involved in signaling networks that regulate cell migration, cytoskeletal rearrangements,

ABBREVIATIONS: EGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; GPCR, G protein–coupled receptor; GST, glutathione-S-transferase; HEK, human embryonic kidney; Hsp90, heat-shock protein 90; IRES, internal ribosomal entry site; LARG, leukemia-associated RhoGEF; MRTP, myocardin–related transcription factor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RBD, Rho-binding domain; RhoGEF, guanine nucleotide exchange factor for Rho; siRNA, small interfering RNA; SRE, serum response element.
adhesion, apoptosis, and growth (Juneja and Casey, 2009; Suzuki et al., 2009). Consistent with their role as signaling hubs in these pathways, overexpressed or constitutively activated Ga12 and Ga13 are potent stimulators of oncogenic transformation (Chan et al., 1993; Jiang et al., 1993; Xu et al., 1994). Some of these responses appear to involve transcriptional activation of growth-promoting genes governed by promoters harboring the serum response element (SRE) (Vara Prasad et al., 1994; Hill et al., 1995; Fromm et al., 1997). Other responses point to cell migration: endogenous Ga12 levels correlate with the degree of metastatic invasiveness in breast cancer tissue samples (Kelly et al., 2006a), and androgen-insensitive, invasive prostate tumor cells exhibit higher Ga12 levels than less aggressive prostate cancers (Kelly et al., 2006b). Despite 65% amino acid identity, Ga12 and Ga13 have nonoverlapping roles in signaling, embryonic development, and disease physiology. Mice lacking Ga13 exhibit embryonic lethality with impairment in vascular development (Offermanns et al., 1997), whereas mice lacking Ga12 appear to develop normally (Gu et al., 2002). However, in mice that are haplo-insufficient for Ga13, at least one wild-type allele of Ga12 is required to avoid lethality (Worzfeld et al., 2008), suggesting that Ga12 function during development is not eclipsed by Ga13. Also, studies of mouse embryonic fibroblasts lacking Ga12 and Ga13 reveal that both proteins are necessary for orientation of the microtubule-organizing center (Goulimari et al., 2008).

The mechanisms through which Ga12 and Ga13 stimulate cell proliferation are not well understood. The best characterized effector proteins are Rho-specific guanine nucleotide exchange factors (RhoGEFs) that interact with Ga12 and Ga13 via RGS (regulator of G protein signaling) homology domains; these include p115RhoGEF, leukemia-associated RhoGEF (LARG), and PDZ-RhoGEF (Sternweiss et al., 2007). In addition to regulating cytoskeletal events such as contraction, this Ga12/13-RhoGEF-Rho axis also stimulates transcription through the SRE, a promoter element of the c-fos protooncogene (Fromm et al., 1997; Bhattacharyya and Wedegaertner, 2000; Shi et al., 2000). This signaling event is mediated by the transcriptional activator serum response factor, which itself requires Rho-mediated nuclear translocation of its cofactor, myocardin-related transcription factor-A (MRTF-A) (Wang et al., 2002). Although Ga12 and Ga13 signal through several common binding partners, numerous studies provide evidence of selectivity within the G12/13 class (Kelly et al., 2007). For example, activity of heat-shock protein 90 (Hsp90) is required for Ga12 but not Ga13, to stimulate cellular transformation (Vaiskunaitė et al., 2001).

Hsp90 is a molecular chaperone that forms a homodimer in the cytoplasm, and hydrolysis of ATP at N-terminal binding pockets within this dimer facilitates engagement and stabilization of a wide variety of Hsp90 client proteins, many of which are implicated in cancer progression (Samant et al., 2012). Hsp90 binds specifically to Ga12 within the G12/13 class, but the structural features that mediate this interaction are unknown.

Recent studies used a taxonomic comparison of Go proteins to identify changes in key residues that contributed to the evolutionary diversification of the four classes (Friedman et al., 2009; Temple et al., 2010). In the present study, we mutated "class-distinctive" residues in Ga12 to ancestral forms to dissect their roles in distinct signaling pathways. Our results revealed a subset of these class-distinctive mutations in Ga12 that disrupted both SRE activation and Hsp90 binding. Ga13 variants harboring identical mutations displayed robust SRE activation, suggesting a Ga12-specific mechanism. In addition, the Drosophila G12/13 homolog Concertina was unable to drive SRE signaling when expressed in mammalian cells, and subsequently we used this protein as a platform to identify key determinants of growth signaling in the switch regions and C-terminal region of Ga12. These findings define Ga12-specific structural determinants of SRE activation and implicate Hsp90 binding as a requirement for this Ga12-mediated pathway.

Materials and Methods

Materials and DNA Constructs. Glutathione-S-transferase (GST) fusions of the N-terminal 252 amino acids of p115RhoGEF and the region spanning Leu320 to Arg606 of LARG were provided by Tohru Kozasa (University of Illinois, Chicago) and have been described previously (Meigs et al., 2005). A GST fusion of the C-terminal 107 amino acids of Hsp90-α was provided by Tatjana Vyno-Yasenetskaia (University of Illinois, Chicago), and enhanced green fluorescent protein (EGFP)-fused MRTF-A was a gift from Christopher Mack (University of North Carolina, Chapel Hill). All point mutants of Ga11, Ga12, and Concertina were engineered by oligonucleotide-directed mutagenesis using the QuikChange II system (Agilent Technologies, Englewood, CO), with the following exception to the manufacturer’s instructions. Each initial amplification reaction was divided into equal halves, with each receiving one of two mutagenic oligonucleotides for the first two polymerase chain reaction (PCR) cycles. These half-reactions were then combined and subjected to 15 additional PCR cycles. Concertina mutants in the internal ribosomal entry site (IRES) vector pLL-5.5 (Uetrecht and Bear, 2009) were engineered by first excising the Concertina cDNA from pLL-5.5 using Apal and EcoRI, subcloning into a truncated pGEX-2T vector (GE Healthcare, Little Chalfont, Buckinghamshire, UK) at these sites and then using QuikChange II reagents to introduce codon substitutions. Mutants were subcloned into pLL-5.5 and then confirmed by sequencing. To introduce myc-Ga13 myc-Ga12-Ga13 cDNA was excised from pcDNA3.1 (Life Technologies, Grand Island, NY), using EcoRI and the blunt end-generating PmeI, and was ligated into pLL-5.5. All DNA constructs expressed in Drosophila cell culture were subcloned into a metallothionein promoter, pmtA vector backbone (Life Technologies), and a myc epitope tag was introduced N-terminally after the initiator Met using KOD Xtreme Hot Start Polymerase (EMD Millipore, Billerica, MA). All Ga12/Concertina and Ga13/Concertina chimeras were engineered by a two-step PCR procedure, in which desired regions of the mutationally activated Ga12 (Q229L), Ga13 (Q226L), and Concertina (Q303L) cDNAs were amplified so that each initial product contained an additional nine base pairs that overlapped with the product amplified from the other cDNA. This created junctions in which 18 base pairs formed, allowing the initial PCR products (two for chimeras 1, 2, 3, and 4, three for all other chimeras) to be combined with end primers in a second round of PCR cycling to generate a full chimeric product. For N-terminal tagging of chimeras 3, 4, 5, and 4-sub, the coding sequence was amplified by PCR with the myc epitope tag EQLIQEEDL encoded in the forward primer immediately downstream of the initiator methionine, and each product was subcloned into pcDNA3.1+ (Life Technologies). All constructs were verified by sequencing.

Expression and Immobilization of GST Fusion Proteins. The GST fusion constructs were transformed into BL21(Gold)-DE3 cells (Agilent Technologies), liquid cultures were grown at 37°C under 75 μg/ml ampicillin selection to OD600 of 0.5-0.7, and recombinant protein expression was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside
Preparation of Detergent-Soluble Extracts Harvesting Go Mutants. Human embryonic kidney cells (HEK293) were grown in Dulbecco’s modified Eagle’s medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), penicillin, and streptomycin. For myc-Go14α and each of the class-distinctive mutants, 7.0 µg of plasmid DNA was transfected into a 10-cm dish of HEK293 cells at approximate 90% confluence, using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer’s instructions. After 36–40 hours, cells were scraped from dishes, washed twice with phosphate-buffered saline (PBS), and solubilized in lysis buffer [50 mM HEPES pH 7.5, 1 mM EDTA, 3 mM dithiothreitol, 10 mM MgSO4, 1% (w/v) polyoxyethylene-10-lauryl ether] containing the protease inhibitors 4-aminophenylmercuric benzenesulfonate fluoride hydrochloride (1.67 mM), leupeptin (2.1 µM), pepstatin (1.45 µM), Na-tosyl-L-lysine chloromethyl ketone (58 µM), tosyl-L-phenylalanyl-chloromethane (61 µM), and phenylmethylsulfonyl fluoride (367 µM). Samples were centrifuged at 80,000g for 1 hour, and supernatants were snap-frozen and stored at −80°C.

Trypsin Protection Assays. HEK293 cells grown in 10-cm dishes were transfected with various Ga14 constructs using Lipofectamine 2000, and tryptic digestions were performed as a modification of the procedure of Kosaka and Gilman (1995). Briefly, cells were lysed in 50 mM HEPES, pH 7.5, 1 mM EDTA, 3 mM dithiothreitol, and 1% polyoxyethylene-10-lauryl ether containing the same protease inhibitors as lysis buffer (see preceding) but at 2-fold lower concentration. Samples were cleared by centrifugation at 80,000g for 1 hour, and supernatants were diluted 20-fold in volume using 50 mM HEPES pH 7.5, 1 mM EDTA, 3 mM dithiothreitol, and 10 mM MgSO4. Samples were digested with 10 µg/ml TPC-treated trypsin (New England Biolabs, Ipswich, MA) for 20 minutes at 30°C, and proteolysis was terminated by addition of 100 µg/ml lima bean trypsin inhibitor (Warthington, Lakewood, NJ) Proteins were precipitated by addition of 20% trichloroacetic acid and 0.8 mg/ml sodium deoxycholate, washed with acetone, dried, and then analyzed by SDS-PAGE and immunoblotting using J169 antisera specific to the Ga14 C terminus (Kosaka and Gilman, 1995), provided by Tokru Kosaka (University of Illinois, Chicago).

Protein Interaction Assays. Extracts from transfected HEK293 cells were diluted in lysis buffer (see preceding description) lacking polyoxyethylene-10-lauryl ether, using sufficient volume to dilute this detergent to 0.05% (w/v). Next, Sepharose-bound GST fusion proteins were added and continuously inverted for 2 hours at 4°C. A percentage of the diluted extract was set aside as starting material (i.e., load) before Sepharose addition. Samples were centrifuged at 1,300g, and pellets were washed extensively and subjected to SDS-PAGE and immunoblot analysis using an antibody specific to either the Ga14 N terminus (Santa Cruz Biotechnology, Santa Cruz, CA), Ga14 (EMD Millipore), or the myc epitope tag (EMD Millipore), followed by alkaline phosphatase conjugated secondary antibodies (Promega, Madison, WI). For each Ga protein, the Gaussian intensity value was determined for the 44-kDa band in the precipitated material and divided by the Gaussian intensity of the corresponding 44 kDa-band in the load to normalize Ga variants for different expression levels in cells.

RhoA Activity Assays. Pull-downs using the Rho-binding domain (RBD) of Rhotekin were performed as previously described (Ren et al., 1999) with the following minor modifications. Cells were washed with ice-cold Tris buffered saline (pH 7.6) with 2 mM MgCl2 and lysed in buffer A (50 mM Tris pH 7.6, 500 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 0.5 mM MgCl2, 200 µM orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Lysates were sonicated briefly, clarified by centrifugation, and equalized for total volume, as well as protein concentration, and then incubated with 30 µg of GST-RBD beads prepared as previously described (Guilluy et al., 2011). Samples were washed with buffer B (50 mM Tris pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.5 mM MgCl2, 200 µM orthovanadate, and protease inhibitors as described) three times before SDS-PAGE. Active RhoA, total RhoA, GTPγS, and actin levels were determined by immunoblotting with anti-RhoA (polyclonal antibody 6789; Cell Signaling Technology, Danvers, MA), anti-Ga14 (Santa Cruz Biotechnology), and anti-actin (clone C4; EMD Millipore). Total RhoA and Ga14 levels were similarly determined using an aliquot of whole cell lysate.

Reporter Gene Assays and RNA Interference. SRE-luciferase plasmid was provided by Channing Der (University of North Carolina, Chapel Hill). HEK293 cells grown to approximately 80% confluence in 12-well plates were transfected with 0.2 µg of SRE-luciferase and 0.02 µg pRL-TK harboring the cDNA for Renilla luciferase (Promega), plus 1.0 µg plasmid encoding a variant of myc-Go14α, Go14α, or Concertina. Plasmid mixtures were combined with Lipofectamine 2000 in Opti-MEM reduced serum medium (Life Technologies) according to the manufacturer’s instructions. For RNA interference experiments, small interfering RNA (siRNA) specific to Hap90a (ON-TARGETplus SMARTpool, Thermo Scientific Dharmacon, Pittsburgh, PA) was reconstituted in 1 µl siRNA buffer (Thermo Scientific Dharmacon), and 60 pmol per sample was combined with the plasmids described above and cotransfected using Lipofectamine 2000 into 12-well plates of HEK293 cells. Assays for SRE activation were performed as described previously (Meigs et al., 2005). Briefly, cells were washed with phosphate-buffered saline and lysed in 1% passive lysis buffer (Promega), and lysates were analyzed using a Dual-luciferase assay system and GloMax 20/20 luminometer (Promega). Light output from firefly luciferase activity was divided by output from Renilla luciferase activity to normalize for variations in transfection efficiency.

Visualization of MRTF-A Localization. Subcellular distribution of EGFP-tagged MRTF-A was assayed as previously reported (Hinson et al., 2007; Medlin et al., 2010) with minor modifications. HEK293 cells grown on glass coverslips to approximately 50% confluence were transfected with plasmids encoding EGFP-MRTF-A and variants of Go14 using Lipofectamine 2000 (Life Technologies). After 36 hours, cells were washed in PBS and fixed in 4% paraformaldehyde for 10 minutes, rinsed three times in PBS, and mounted on 4',6-diamidino-2-phenylindole–Fluoromount-G (Southern Biotech, Birmingham, AL). Cells were visualized on a TE-2000S (Nikon, Melville, NY) equipped with a SPOT monochrome digital camera (Diagnostic Instruments, Sterling Heights, MI). Individual cells were examined for 4',6-diamidino-2-phenylindole fluorescence to define the nuclear compartment, followed by green fluorescence to visualize EGFP-MRTF-A distribution.

Drosophila Cell Culture and Imaging. S2 cells were obtained and cultured as previously described (Rogers and Rogers, 2008). Cells were maintained in SF900 media (Life Technologies) and were transfected with 2 mg/ml DNA using the Amaxa nucleofector system (Lonza, Basel, Switzerland) with KitV and program G-50. Cells were plated onto ConA-coated coverslips in petri dishes and induced with 1 mM CuSO4 for 2.5 hours. Cells were prepared for imaging as previously described (Rogers and Rogers, 2008). Probes used were 1:400 diluted anti-myc (9E10) antibody (DSHB, Iowa City, IA) and 1:100 diluted Alexa Fluor 488 phallolidin (Life Technologies). Cells were imaging using an Eclipse Ti-E (Nikon).

Data Analysis and Statistics. Immunoblot results were quantified using a Kodak Gel Logic 100 imaging system equipped with Molecular Imaging 5X software (Carestream Health, New Haven, CT) to calculate Gaussian fit for each protein band. For graphical data presentation, error bars represent either ± range or ± S.E.M., as indicated in the figure legends (Figs. 1-7). Student’s t test with unequal variance was used to compare the means of two data sets with one measurement variable, and the Kruskal–Wallis test was.
used for one-way analysis of variance. A P value < 0.05 was considered significant.

**Results**

**Identification and Mutation of Class-Distinctive Residues within Gα12.** Each Gα class is thought to signal to its downstream effectors via specialized contact surfaces that are ultimately determined by primary sequence. Therefore, we hypothesized that these effector-binding surfaces harbor unique, “class-distinctive” amino acids that confer specificity for downstream signaling partners. To identify G12/13 class-distinctive residues, we conducted a structural alignment of all four classes of mammalian G protein α-subunits: Gαs, Gαi, Gαq, and G12/13 as described by Temple et al. (2010) and identified 19 class-distinctive residues in the G12/13 class. Of these residues, 16 were located either in Gα12 alone or in both Gα12 and Gα13 and were selected for further study. Through ancestral reconstruction analyses, we determined that the “nonclass-distinctive” counterpart, represented by a residue invariant among the other three mammalian Gα classes, most frequently represents the ancestral amino acid value (Temple et al., 2010). We engineered substitutions within a myc-tagged, constitutively active variant (Q229L) of Gα12, termed myc-Gα12QL, with each mutation converting a class-distinctive residue to its nonclass-distinctive form (Fig. 1A). Each mutant was engineered as a single substitution, with the exception of Q232E/Q234K in which two Gln residues were changed in the same construct, yielding a total of 15 Gα12 variants. When expressed in HEK293 cells, nearly all class-distinctive mutants were detected at levels comparable to unaltered myc-Gα12QL (Fig. 1B). Endogenous Gα12 migrated slightly faster on gels than the myc-tagged variants and was not detectable (Fig. 1B, vector) except when immunoblots were developed for relatively long times.

**Class-Distinctive Gα12 Mutants Selectively Uncoupled from SRE Activation and Hsp90 Binding.** We examined the Gα12 class-distinctive mutants for activation of SRE-mediated transcription, a Rho-dependent cell growth pathway that is responsive to Gα12 and Gα13 (Fromm et al., 1997; Bhattacharyya and Wedegaertner, 2000). As shown in Fig. 2A, most of these mutants displayed normal or moderate impaired ability to stimulate a SRE-luciferase reporter construct (>50% of control, nonmutated myc-Gα12QL); however, the mutant L201R and the double-mutant Q232E/Q234K showed severe impairment of SRE stimulation (<20% of control), whereas the mutant F237I showed near-complete loss of SRE activation that was comparable to a constitutively inactive mutant (G228A) of Gα12 that is unable to release GDP (Miller et al., 1988). We further tested these SRE-uncoupled Gα12 mutants for an additional readout in this signaling pathway, nuclear translocation of MRTF-A/megakaryoblastic leukemia 1. This trafficking is mediated by activated Gα12 and Gα13 and requires downstream Rho activation, which participates in mediating MRTF-A nuclear import and activity by decreasing the availability of the MRTF-A binding partner, G-actin (Evelyn et al., 2007; Medjkane et al., 2009). As shown in Fig. 2B, cells expressing EGFP-tagged MRTF-A and cotransfected with myc-Gα12QL showed a higher percentage of cells with exclusively nuclear EGFP-MRTF-A staining than cells cotransfected with the inactive (G228A) myc-Gα12. Although the SRE-uncoupled mutants of myc-Gα12QL (L201R, Q232E/Q234K, F237I) showed varied results in this assay, all exhibited a lower percentage of cells with nuclear EGFP-MRTF-A localization than the positive control myc-Gα12QL, suggesting the impaired ability of these mutants to signal to SRE involves a mechanism upstream of MRTF-A transport to the nucleus.

In parallel to the SRE activation studies shown in Fig. 2A, we examined whether mutation of class-distinctive residues in Gα12 to their nonclass-distinctive forms disrupted interaction with specific effector proteins. Myc-tagged Gα12QL and its mutants were expressed in HEK293 cells and tested for the ability to bind GST fusions of p115RhoGEF, LARG, and Hsp90 (Fig. 3A). As shown in Fig. 3B, each of these proteins was able to precipitate myc-Gα12QL from cell extracts.

**Fig. 1.** Construction, expression, and solubilization of class-distinctive Gα12 mutants. (A) Amino acid sequence of Gα12 is shown. Each class-distinctive residue, indicated by an oval, was mutated to its nonclass-distinctive form (above right of ovale) in myc-tagged, constitutively active Gα12. Shaded areas indicate the switch I, II, and III regions, and the dashed box indicates the site of the activating Q229L mutation. (B) The indicated mutant constructs were expressed in HEK293 cells, from which detergent-soluble extracts were subjected to immunoblot analysis as described in Materials and Methods. All mutants were single amino acid substitutions except the double-mutant Q232E/Q234K (QE/QK). Extracts from cells transfected with the Q229L variant of myc-tagged Gα12 (J2QL) and empty pcDNA3.1 (vector) were analyzed in parallel.
Fig. 2. Identification of class-distinctive mutants of Go12 impaired in SRE-mediated transcriptional activation. (A) HEK293 cells were transfected with the plasmids SRE-luciferase (0.2 µg) and pRL-TK (0.02 µg) plus 1 µg of plasmid encoding each indicated class-distinctive mutant of myc-Go12C (x-axis). Firefly luciferase activity values were normalized for Renilla luciferase activity values and presented as a percent of positive control myc-Go12C (12Q) within the same experiment. Empty pcDNA3.1 (vector) and Go12 harboring the inactivating G228A mutation (12GA) were examined as negative controls. Data presented are the mean ± range of two independent experiments per myc-Go12 variant, with three or more experiments performed for mutants that showed greater than 50% impairment of SRE stimulation. (B) HEK293 cells grown in six-well plates were cotransfected with 1.0 µg of EGFP-MRTF-A plasmid and 1.0 µg of each indicated Go12 construct. Cells (50 per transfection) were scored for either diffuse or exclusively nuclear localization of the EGFP signal, using 4′,6-diamidino-2-phenylindole (DAPI) containing to define the boundaries of the nucleus. Examples are shown in the left panels (two cells exhibiting diffuse EGFP-MRTF-A staining) and right panels (single cell exhibiting nuclear staining of EGFP-MRTF-A). Scale bar is 10 µm. The column graph shows results compiled from three independent trials, presented as mean ± S.E.M. Significance of the difference in mutant values compared with positive control (12Q) was determined by Student’s t test (P < 0.05).

Among the class-distinctive mutants, L201R, F237I, and Q232E/Q234K were most severely impaired in binding Hsp90, with a precipitate-to-load ratio between 0 and 20% of the value for myc-Go12C (Fig. 3C; Table 1). This indicated that the subset of Go12 class-distinctive residues most indispensable for stimulating SRE-mediated transcription are also the most important in Go12-Hsp90 interaction. These mutations that attenuated SRE signaling and Hsp90 binding did not globally affect Go12 interaction with downstream targets, as all three mutants bound strongly to LARG and p115RhoGEF (Fig. 3D; Table 1). Furthermore, these mutations did not disrupt Go12 structural conformation. As shown in Fig. 3E, the mutants L201R, F237I, and Q232E/Q234K yielded a trypsin-protected 40-kDa fragment comparable to the fragment generated from nonmutated myc-Go12C, whereas the inactive G228A variant of myc-Go12 was fully digested. Therefore, it appears these Go12 mutants retain fundamental properties of guanine nucleotide binding, and their impairment in SRE stimulation and Hsp90 binding appears not to be caused by failed conformational activation or other, non-sequence disruptions of protein folding or effector binding.

Phylogenetic Substitutions of Class-Distinctive Go12 Residues Have Differential Effects on SRE Signaling. In addition to comparing the different mammalian Go classes to identify residues that confer signaling specificity, we determined the phylogenetic timeline of G12/13 class-distinctive residues by comparing divergent sequences from sea sponge, roundworm, fruit fly, sea urchin, and mouse or human (Temple et al., 2010). Evolutionary emergence of the
Leu residue immediately upstream of the switch I region (Leu201 in Gα12, Leu198 in Gα13) was relatively recent for the G12/13 class because a His residue occupies this position in the sea urchin and Drosophila homologs, and the Caenorhabditis elegans and sea sponge G12/13 proteins harbor other residues (Fig. 4A). In contrast, Phe237 in the switch II region of Gα12 is highly conserved in G12/13 proteins of all the taxa examined (Fig. 4A) except Drosophila, in which Thr occupies this position. To assess the structural and functional consequences of these evolutionary changes within the G12/13 class, we mutated Leu201 and Phe237 in myc-Gα12QL to the corresponding Drosophila residues His and Thr, respectively. The L201H mutant (i.e., mammal fly) exhibited normal SRE activation and binding to Hsp90, in sharp contrast to L201R (i.e., class-distinctive nonclass-distinctive) that caused impairment of both functions (Fig. 4, B and C). Surprisingly, the F237T mutant (mammal fly) in myc-Gα12QL showed near-complete loss of SRE stimulation (Fig. 4B), phenocopying the Phe237 mutation to Ile (class-distinctive nonclass-distinctive), and both F237I and F237T mutants were markedly impaired in Hsp90 binding (Fig. 4C). For all variants of residues Leu201 and Phe237 that we engineered, binding of Gα12 to LARG was unperturbed or slightly diminished (Fig. 4C). In some experiments, protein levels of mutants L201R and F237I were lower than the positive control myc-Gα12QL; therefore, to ascertain that impaired SRE signaling was due merely to lowered expression of these mutants, we examined a series of cell samples transfected with decreasing amounts of myc-Gα12QL plasmid (Fig. 4B). Mutants F237I and L201R showed impaired SRE activation compared with myc-Gα12QL expressed at similar levels. From these data, we conclude that Leu201 and Phe237 in mammalian Gα12 were critical components of its evolved ability to stimulate SRE and bind Hsp90, but the corresponding residues in the Drosophila G12/13 homolog Concertina represent different stages of this process. The His residue upstream of the switch I region in Concertina is effectively a G12/13 class-distinctive residue (i.e., facilitated the same signaling properties as Leu201 in Gα12) and therefore might be an intermediate to fixation of the Leu residue in mammalian G12/13 proteins. On the other hand, the Thr residue in the switch II region of Concertina fails to act as a G12/13 class-distinctive residue; its introduction to Gα12 disrupted both SRE-mediated signaling and Hsp90 binding. It remains to be determined whether this Thr residue confers a unique signaling property in Concertina.
despite the conservation of Phe at this position in the G12/13 class from sea sponge to humans. To our knowledge, these results provide the first instance of a signaling function being disrupted in a mammalian G12/13 protein by substitution of corresponding sequence from an evolutionary homolog in the same class.

Structural Determinants of Hsp90 Interaction Are Selectively Required for Go_{12} in G12/13-Mediated SRE Activation. Go_{13}, like Go_{12}, is a potent stimulator of SRE-mediated transcriptional activation (Fromm et al., 1997), and activated mutants of both Go_{12} and Go_{13} are blocked in SRE activation by the Clostridium botulinum C3 exoenzyme, a specific inhibitor of Rho activity (Shi et al., 2000). To assess the requirement for Hsp90 function in Go_{12} and Go_{13} signaling, we used a constitutively active (Q226L) variant of Go_{13} along with constitutively active Go_{12} and examined effects of the Hsp90 inhibitor geldanamycin on SRE activation. Hydrolysis of ATP is critical for Hsp90 in forming a “clamped” complex with client proteins, and geldanamycin disrupts this biologic function of Hsp90 by competing for ATP binding to the N-terminal domain (Peterson and Blagg, 2009). We found geldanamycin to partially block Go_{12}-mediated activation of SRE-luciferase in HEK293 cells, whereas this compound showed significantly lower efficacy in blocking Go_{13} stimulation of this reporter (Fig. 5A). This selectivity of geldanamycin on Go_{12} signaling within the G12/13 class agreed with previous findings in NIH3T3 fibroblasts (Vaiskunaite et al., 2001). In addition, we tested the ability of Go_{12} and Go_{13} to stimulate SRE-luciferase in HEK293 cells cotransfected with siRNA targeting human Hsp90α. These experiments yielded results similar to the effects of geldanamycin; this RNA interference caused significantly greater downregulation of Go_{12}-mediated SRE activation in comparison with the Go_{13}-driven response (Fig. 5A). To determine whether the SRE-uncoupled mutants define a mechanism specific to Go_{12} within the G12/13 family, we engineered constitutively active Go_{13} to harbor substitutions of Leu198 and Phe234 to their nonclass-distinctive forms (mutants L198R and F234I) and their Concertina-specific forms (mutants L198R and F234T). In striking contrast to Go_{12}, these substitutions in Go_{13} caused no disruption in SRE signaling (Fig. 5B). We also examined the role of Go_{13} residues that correspond to the class-distinctive Gln232/Gln234 pair found in Go_{12}. As described already herein (Figs. 2 and 3), the Q226E/Q234K mutant converts Gln residues within the Go_{12} switch II region to their nonclass-distinctive forms, disrupting SRE stimulation and Hsp90 binding. Paradoxically, these nonclass-distinctive Gln and Lys residues are present in Go_{13}, despite conservation of the Gln/Gln pair in the G12/13 class for all nonmammalian taxa examined (Fig. 4A). We hypothesized that “reversion” from Gln/Gln to the ancestral Gln/Lys was necessary for Go_{13} to evolve its distinct mechanism of growth signaling, and therefore we mutated Gln229 and Lys231 in Go_{13} to a pair of Gln residues and tested this E229Q/K231Q mutant in SRE-luciferase assays. Surprisingly, these substitutions caused no disruption of SRE activation triggered by constitutively active Go_{13} (Fig. 5B), suggesting that Gln229 and Lys231 of Go_{13} are uninvolved in its stimulation of SRE-mediated transcription, whereas Gln residues at these positions in Go_{12} are critical for its mechanism of activating the same response. A structural comparison of Go_{12} to Go_{13} in the switch II region reveals similarities and differences in backbone and side-chain atoms at the Q232E/Q234K positions (Fig. 5C). In Go_{12}, the Q232E substitution alters only the charge of the side-chain, whereas the Q234K substitution changes both size and charge. Taken as a whole, these findings suggest that the effector proteins stimulated by Go_{12} and Go_{13} in the pathway(s) leading to SRE-mediated transcription are not identical and that interaction with functional Hsp90 is a specific requirement of Go_{13} in this signaling response.

Class-Distinctive Mutants of Go_{12} Uncouple SRE Activation from Rho-Mediated Signaling. Our results, shown in Fig. 4, revealed a single amino acid substitution (F237T; mammalian fly) that uncoupled Go_{12} from SRE activation, and this finding compelled us to examine whether the Drosophila Go_{12} homolog Concertina could stimulate the SRE pathway when expressed in mammalian cells. Concertina is required for cell shape changes and migration during gastrulation (Parks and Wieschaus, 1991), but its growth signaling properties are not known. Because Concertina-specific antibodies were not available, we subcloned both wild-type and constitutively active Concertina (both Q303L and R277C variants) into the mammalian cell expression vector pLLS.5 (Uetrecht and Bear, 2009) harboring an IRES to allow translation from a bicistronic mRNA, along with green fluorescent protein (GFP). Neither wild-type nor constitutively active Concertina (Q303L or R277C) stimulated SRE-luciferase when expressed ectopically in HEK293 cells, despite the expression of GFP (Fig. 6A). We were not able to obtain a definitive result in experiments testing ability of the Q303L variant of Concertina to trigger a cytoskeletal response in HEK293 cells (data not shown); however, this Q303L variant was functional in its normal cellular context as an activator of contractility in S2 cells (Fig. 6, B and C), as was the R277C variant (data not shown). As a result of the acute disruption of Go_{12}-mediated SRE signaling observed when Phe237 was converted to the Concertina-specific Thr, we engineered the converse mutant by replacing Thr311 of Concertina with the Go_{12}-specific Phe residue. However, this T311F substitution did not confer on Concertina the ability to activate SRE-mediated transcription in HEK293 cells (Fig. 6A).

Of the class-distinctive Go_{12} residues we identified as critical for SRE activation, only the switch II region Gln/Gln pair is conserved between Go_{12} and Concertina. To determine whether these Gln residues are important in Concertina-driven cytoskeletal rearrangements, which is a signaling pathway known to use the RhoGEF-Rho axis (Barrett et al., 1997), we tested a Q306E/Q308K mutant of constitutively active Concertina (Q303L) for the ability to stimulate S2 cell contraction. This Concertina mutant showed no loss of efficacy in triggering contractility of S2 cells (Fig. 6, B and C), suggesting that these Gln residues are not involved in the Go-driven mechanism that mediates these Rho-dependent cytoskeletal changes. We also directly examined the SRE-uncoupled Go_{13} mutants (L201R, F237I, and Q232E/Q234K) for Rho activation, by using RBD assays to selectively precipitate GTP-bound RhoA from HEK293 cell lysates. As shown in Fig. 6D, the F237I and L201R mutants showed activation of Rho comparable to positive control myc-Go_{12}QL. The Q232E/Q234K mutant of myc-Go_{12}QL triggered a low, although significant, activation of Rho in comparison with inactive, GDP-bound myc-Go_{12}, despite the robust cytoskeletal response triggered in S2 cells by Concertina harboring the same substitutions.
**Ga₁₂ Determinants of SRE Activation**

Because Concertina failed to stimulate SRE-mediated transcription in HEK293 cells, we used this *Drosophila* Ga₁₂ homolog as a “blank canvas” in these mammalian cells for identifying regions of Ga₁₂ that mediate SRE activation. We engineered a set of Ga₁₂/Ga₁₃ chimeras, designated numbers 1–6, in which a domain encompassing the three switch regions and the flanking N- and C-terminal domains were interchanged in all possible combinations (Fig. 7A). Each chimera was engineered to harbor an activating Gln-to-Leu mutation within the switch II region and was subcloned into the IRES vector pLL-5.5 to allow normalization of expression through GFP immunoblotting (Uetrecht and Bear, 2009). Strikingly, the only construct that stimulated SRE signaling was chimera 4, which harbors the N-terminal 275 residues of Concertina and an additional 178 residues comprising the switch regions and C terminus of Ga₁₂ (Fig. 7B). Whereas this chimera showed robust SRE activation equal to or greater than myc-Ga₁₂, the other chimeras exhibited no SRE stimulation, with readings comparable to mutagenically activated Concertina or the IRES vector expressing GFP only (Fig. 7B). GFP levels in cell lysates were similar, suggesting that the bicistronic mRNAs encoding all six chimeras were comparable in levels and translation rate (Fig. 7B). These results indicated the switch regions and C-terminal domain of Ga₁₂ harbor determinants that are independently required for SRE activation in HEK293 cells because replacement of either domain with Concertina sequence abrogated this response. To define determinants within the Ga₁₂ C terminus critical for this signaling function, we aligned the Ga₁₂ and Concertina C-terminal domains and found two regions of close homology, separated by a divergent region spanning Lys304 to Phe345 in Ga₁₂ and Cys379 to Tyr423 in Concertina (Fig. 7A). To determine whether this region harbors Ga₁₂-specific determinants of SRE activation, we engineered a “sub-chimera” (designated chimera 4-sub) in which this Ga₁₂ region in chimera 4 was replaced by Concertina sequence. Because N-terminal epitope-tagging of Concertina did not disrupt its ability to stimulate S2 cell contraction (Fig. 6, B and C), we engineered an N-terminal myc tag in chimeras 4 and 4-sub and similarly tagged the other chimeras that harbored the Concertina N-terminal domain (chimeras 3 and 5). As shown in Fig. 7C, chimera 4-sub displayed near-complete loss of SRE signaling, even though immunoblots showed similar expression levels to chimera 4. Myc-tagged chimeras 3, 4, and 5 generated essentially the same SRE-luciferase readouts as their untagged forms (data not shown). We also generated a variant of myc-Ga₁₂ (Δ), designated Δ45, harboring a substitution that introduced the same C-terminal 45-residue Concertina sequence found in chimera 4-sub, and as predicted, this mutation caused a complete loss of SRE activation (Fig. 7C). The corresponding Δ45 mutant of Ga₁₃ (Δ), in which its region spanning Gln301 to Tyr343 was replaced by the...
Fig. 6. Differential effects of Ga12 and Concertina on SRE-mediated transcription and cytoskeletal rearrangements. (A) Effect of Concertina variants on SRE-mediated transcriptional activation. HEK293 cells were transfected with a GFP-encoding IRES vector harboring myc-Ga12a (12QL), no additional coding sequence (none), or Concertina variants with the following designations: wt, wild-type; RC, R277C; QL, Q302L; TP, T311F. For each sample, luciferase assays were performed (graph) and GFP levels were determined by immunoblot analysis (inset) to allow indirect measure of expression of Concertina variants. Data shown are representative of three independent experiments. (B) Effect of Concertina and its Q306E/Q308K variant on cell contraction. S2 cells expressing inducible myc-tagged, constitutively active Concertina (CtaQL) or the same protein harboring Q306E/Q308K substitutions (QE/QK) were induced with 50 μM copper sulfate and 24 hours later scored for contractility as described in Materials and Methods. Cells were stained with phalloidin (top row) and anti-myc antibody (middle row), and images were merged (bottom row). Scale bar is 10 μm, and images are from a representative of three independent experiments. (C) Compiled quantitative data from experiments described in (B) are shown, with bars indicating S.E.M. (D) HEK293 cells were assayed for basal RhoA activity 36–42 hours after transfection with constitutively active (12GA) or inactive (12GA) myc-tagged Ga12a, or the indicated mutants of myc-Ga12a. Blots were developed with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) and Kodak Biomax film, and representative blots of active RhoA (Rho-GTP) precipitated by GST-RBD versus total RhoA in cell lysates are shown, along with blots of Ga12 and actin in the same experiment. RhoA activity is the ratio of active RhoA to total RhoA signal, with densitometry (NIH ImageJ software) used to quantify results from seven independent experiments. Results are graphed as the fold increase over cells expressing inactive, GFP-bound Ga12a (12GA). Graphical data represent mean ± S.E.M.; **P < 0.001 as calculated through analysis of variance using the Kruskal–Wallis test.

The aforementioned Concertina sequence, exhibited robust SRE stimulation (Fig. 7C). These findings bolster our results shown in Fig. 5, suggesting that Ga12 and Ga13 use different structural features and effector binding events in stimulating this cell proliferative pathway. Taken as a whole, our results define specific residues in the switch regions, as well as a region near the C terminus, that differ between Ga12 and its Drosophila homolog and are required for SRE signaling by Ga12, but not Ga13.

Discussion

To illuminate the structural features of Ga12 and Ga13 that mediate their signaling functions, a useful approach has been to test mutants for disruption of cellular responses or binding to downstream targets (Jones and Gutkind, 1998; Adarichev et al., 2003; Nakamura et al., 2004; Vazquez-Prado et al., 2004; Grabocka and Wedegaertner, 2005). Such studies have revealed Ga12 determinants of binding to effectors that include RhoGEFs, protein phosphatase-2A, and polycystin-1 (Meiga et al., 2005; Zhu et al., 2007; Yu et al., 2011). Our current study was guided by a method that identified “class-distinctive” residues within each Ga protein and deduced the ancestral, “nonclass-distinctive” residue at each position (Temple et al., 2010). We engineered a panel of Ga12 mutants by converting each class-distinctive residue to its nonclass-distinctive counterpart; these substitutions were intended to render features of Ga12 as ancestral-like conformations that might lack ability to engage specific targets. These mutants revealed Ga12 residues required for binding to Hsp90 but not RhoGEFs: Len201 just upstream of the switch I region and Phe237 and Gln232/ Gln234 within the switch II region. Unexpectedly, these mutations matched the subset of class-distinctive substitutions we identified as disruptive to Ga12 stimulation of SRE-mediated transcription. The effects of geldanamycin, which inhibits ATP binding to the Hsp90 homodimer and would be predicted to disrupt its functional interaction with client proteins that include Ga12, suggest a relationship between Ga12–Hsp90 interaction and SRE activation, and this hypothesis is further supported by our results after siRNA-mediated targeting of Hsp90 expression (Fig. 5). However, the incomplete effect of geldanamycin on Ga12-stimulated SRE signaling leaves open the possibility that the aforementioned class-distinctive mutations disrupt SRE signaling by hindering Ga12...
interaction with other, unexamined binding partners in addition to Hsp90.

Go12 signaling to SRE requires activation of Rho (Fromm et al., 1997). Because the SRE-uncoupled Go12 mutants exhibited normal binding to RhoGEFs, as well as robust Rho activation for mutants L201R and F237I, these class-distinctive Go12 residues may mediate engagement of an effector pathway(s) required in addition to the canonical RhoGEF-Rho pathway for SRE activation. Results for the Q232E/Q234K mutant of Go12 were less clear; these substitutions disrupted SRE activation and also diminished Rho activation, even though RhoGEF binding remained intact. It is possible this mutation disrupts SRE signaling by partially uncoupling Go12 from the RhoGEF-Rho signaling pathway. However, our finding that Concertina harboring the same mutation triggered normal Rho-mediated contractility in S2 cells (Fig. 6) suggests that this conserved Gln/Gln pair is not required for the ancient G12/13-RhoGEF-Rho axis that mediates cell shape changes in organisms such as Drosophila and C. elegans (Barrett et al., 1997; Yau et al., 2003). Our overall results suggest the L201R and F237I mutants are more decisive than the Q232E/Q234K mutant in selectively uncoupling Go12 from Hsp90 without perturbing Go12-RhoGEF interaction. It is possible this Gln/Gln pair plays a Go12-specific role in mediating Rho binding to a target protein (e.g., Hsp90) that must be engaged for RhoGEF-Rho signaling to commence. The Drosophila Go12 homolog Concertina may lack this requirement, as its Q306E/Q308K mutant was unimpaired in triggering cytoskeletal rearrangements. Taken as a whole, our results suggest the class-distinctive residues essential for Go12-specific SRE activation either mediate a Rho-independent signaling pathway or participate in an effector binding event that facilitates coupling of Go12 to the RhoGEF-Rho pathway.

Both Go12 and Go13 stimulate SRE-mediated transcription via mechanisms that are sensitive to Rho inhibition (Fromm et al., 1997; Bhattacharyya and Wedegaertner, 2000; Shi et al., 2000). However, Hsp90 perturbation preferentially hindered SRE activation by Go12 in comparison with Go13 (Fig. 5), suggesting that nonredundant signaling mechanisms evolved within the G12/13 class. Geldanamycin inhibits thronbin-mediated signaling through protease-activated receptor 1 in mouse neuroblasts but fails to inhibit LPA-mediated signaling (Pai and Cunningham, 2002), and because the thrombin receptor preferentially couples to Go13 whereas LPA signaling uses Go13 (Yamaguchi et al., 2003), this finding implicates Hsp90 in a Go13-specific role. Furthermore, our mutations of residues Leu201 and Phe237 disrupted Go12 but
not Gα13 in stimulating SRE-mediated transcription, despite conservation of these amino acids in both proteins. Another apparent Gα12 determinant of SRE activation, the Gln232/Gln234 pair, is conserved throughout taxa harboring the G12/13 class, whereas the Ga, Gi, and Gq classes use Glu/Lys at these positions. Therefore, it is intriguing that Gα13 "reverted" to the ancestral Glu/Lys pair during its evolution and that the Gln/Gln pair is critical for Gα12 in SRE activation, whereas Gα13 utilizes a signaling mechanism unaffected by Glu/Lys mutation to Gln/Gln. The functional significance of this Glu/Lys motif in Gα13 remains to be determined. Substitution of this Glu residue in Gα13 (Glu229) for a positively charged residue disrupted its RhoGEF binding, SRE activation, and recruitment of p115RhoGEF to the plasma membrane. Conversely, mutation of Glu229 to Ala did not disrupt RhoGEF binding by Gα13, and effects on SRE signaling were not reported (Grabocka and Wedegaertner, 2005). Disruption of SRE signaling by charge reversal at this site may be due solely to interference with RhoGEF engagement, whereas E229Q substitution in our study allowed Gα12 to retain SRE activation and presumably functional RhoGEF binding.

Because Gα12 and Gα13 both stimulate SRE-mediated transcription, albeit through nonredundant mechanisms, the inability of the closely related Concertina to activate this pathway in HEK293 cells was surprising. It is possible that requisite downstream effector proteins in mammalian cells are not engaged by Concertina or that this fly protein fails to fold properly in these cells. However, our data from Gα12/Concertina chimeras (Fig. 7) demonstrate that the N-terminal 275 amino acids, as well as a 45-residue C-terminal region of Concertina, can fold correctly and facilitate robust Gα12- or Gα13-mediated signaling in HEK293 cells. These results suggest that key growth-signaling properties, including mechanisms potentially involved in oncogenic transformation, evolved in the lineage that yielded mammalian G12/13 proteins after divergence from Concertina. Moreover, because Concertina activates cytoskeletal rearrangements in Drosophila through a RhoGEF (DHRGEF2) homologous to the G12/13-coupled mammalian RhoGEFs (Barrett et al., 1997), the striking difference in Gα12 and Concertina signaling to SRE in HEK293 cells bolsters the hypothesis that Gα12 stimulation of this response requires additional effector(s) in these cells besides the canonical RhoGEF-Rho axis. These findings compelled us to interchange residues between Gα12 and Concertina, revealing F237T mutation in Gα12 as disruptive to SRE activation in HEK293 cells, whereas replacement of the Gα12 residue Leu201 with the Concertina residue His had no apparent effect. These results suggest Concertina harbors some, not all, of the structural features necessary for SRE activation in mammalian cells and that such characteristics were partially in place in a common ancestor of Concertina and the mammalian G12/13 proteins. Furthermore, Concertina provides a uniquely useful "null" for Gα12 in our study, due to its 55% identical amino acid sequence and shared signaling properties in regulating Rho-mediated cytoskeletal rearrangements. Our experiments utilizing Gα12/Concertina chimeras revealed both the domain encompassing the switch regions and a span near the Gα12 C terminus as harboring independent determinants of SRE activation. Furthermore, our studies using a corresponding Gα12/Concertina chimera suggested that the same C-terminal span in Gα13 is not involved in its signaling toward SRE activation. Future comparison of G12/13 proteins with these and other chimeras, in assays of effector binding and cellular signaling readouts, may reveal structural features and mechanisms used by Gα12 and Gα13 to activate proliferation, migration, and other responses.

A question that arises from our findings is: what advantage was gained by Gα12 and Gα13 evolving different mechanisms for stimulating SRE-mediated transcription? In considering this question, it is noteworthy that similar examples have been reported in the G12/13 class. Both Gα12 and Gα13 stimulate Na+/H+ exchange across the plasma membrane; however, Gα12 requires protein kinase C, whereas Gα13 uses a pathway independent of this kinase (Dhanasekaran et al., 1994). Gα13 stimulates activity of the Na+/H+ exchangers NHE-1, NHE-2, and NHE-3, but Gα12 stimulates only the latter two proteins while inhibiting NHE-1 (Lin et al., 1996). Also, Gα12-mediated regulation of tight junctions and paracellular permeability occurs via a Src-dependent mechanism, whereas the Gα13-mediated response does not require Src (Meyer et al., 2003; Donato et al., 2009). In another example, recruitment of p115RhoGEF to the inner face of the plasma membrane by activated Gα12 is dependent on RhoA, whereas recruitment by activated Gα13 is refractory to RhoA inhibition (Bhattacharyya et al., 2009). One clue regarding different Hsp90 requirements of the G12/13 proteins has emerged from studies of lipid rafts; Gα12, but not Gα13, localizes to these sphingolipid- and cholesterol-enriched areas, and targeting of Gα12 to lipid rafts is sensitive to Hsp90 inhibition (Waheed and Jones, 2002). The different subcellular locations of Gα12 and Gα13 may facilitate differences in signaling inputs, downstream effectors, and regulators of their respective signaling properties, so that a binding partner such as Hsp90 may be critical for one G12/13 protein but not the other in driving a common downstream response such as SRE activation.

In addition to illuminating target sites for inhibiting Gα12-mediated signaling to SRE-regulated genes, our findings validate the "class-distinctive" methodology (Temple et al., 2010) as a technique that could be extended to other Ga classes to dissect the roles of different binding partners. With the increasing list of proteins that interact with Gα12, Gα13, or other Ga proteins, class-distinctive mutants should be useful for identifying connections between specific Ga-effector interactions and cellular responses mediated by different classes of Ga proteins.

Acknowledgments

The authors thank Tohru Kozasa, Tatyanta Voevo-Yasenetskaya, Channing Der, and Christopher Mack for providing reagents, and Dan Kaplan, Pat Casey, and Aisha Chow for helpful discussions.

Authorship Contributions

Participated in research design: Montgomery, Temple, Peters, Tolbert, Rogers, Jones, Meigs.

Conducted experiments: Montgomery, Peters, Tolbert, Smolski, Hamilton, Tagliatela, Rogers, Meigs.

Contributed new reagents or analytic tools: Montgomery, Peters, Booker, Martin, Hamilton, Tagliatela, Rogers, Meigs.

Performed data analysis: Montgomery, Temple, Peters, Tolbert, Rogers, Jones, Meigs.

Wrote or contributed to the writing of the manuscript: Montgomery, Temple, Peters, Tolbert, Rogers, Jones, Meigs.


