University of North Carolina Asheville
Journal of Undergraduate Research
Asheville, North Carolina
December 2018

Chimeric Analysis of Gα12 Structure: A Divergent C-Terminal Region Provides a Unique Effector Binding Surface

Courtney R. Quick
Department of Biology
The University of North Carolina Asheville
One University Heights
Asheville, NC 28804 USA

Faculty Advisor: Thomas E. Meigs, PhD.

Abstract

The G12/13 class of heterotrimeric guanine-nucleotide binding proteins (G proteins) convert extracellular signals to intracellular responses including cell growth, oncogenic transformation, migration, and cytoskeletal rearrangement. Mammals possess two distinct alpha subunits within this G protein class, Gα12 and Gα13. Sequence divergence after duplication of the ancestral G12/13 gene has led Gα12 and Gα13 to evolve unique binding interactions with various target proteins in the cell such as Hsp90, ARAF, and Axin. These distinct effector interactions have allowed Gα12 and Gα13 to develop unique mechanisms for cell growth signaling through the serum response element pathway. Previous experiments revealed that a variable 42-residue region at the C terminus was necessary for growth signaling in Gα12 but not Gα13. In order to further investigate the functional role of this region in Gα12, several chimeric Gα13 proteins were constructed to contain Gα12 sequence at regions of interest. Protein co-precipitation assays revealed that introducing the variable 42-residue region of Ga12 bestowed Gα13 with the ability to bind to Gα12-specific effector proteins. This finding suggests that sequence divergence in the variable region has allowed Gα12 to evolve distinct functional differences in effector binding that may contribute to its unique mechanism of growth signaling. Because certain cancers selectively overexpress Gα12 or Gα13, our further characterization of this region of Gα12 can be used to guide the development of Gα12-specific growth signaling inhibitors.

1. Introduction

In order to receive and respond to environmental changes, cells have a variety of pathways capable of transducing extracellular chemical information into a diverse number of intracellular events. These signaling pathways are generally initiated by ligands binding to and activating membrane-bound receptors. Conformational change in the receptor upon ligand binding leads to the activation of secondary signaling proteins which transmit the signal to target proteins within the cell. G protein-coupled receptors (GPCR) are integral membrane proteins that respond to a variety of ligands including olfactory stimulatory molecules, hormones, and neurotransmitters[10]. GPCRs transmit signals to a membrane-tethered heterotrimeric G protein complex consisting of an alpha subunit and a beta-gamma dimer. Upon ligand binding to a GPCR, the alpha subunit of the heterotrimer releases GDP and enters an activated, GTP-bound state in which it is separated from the beta/gamma dimer[1] and capable of interacting with downstream effector proteins. There are four classes of alpha subunits, Gs, Gi, Gq, and G12, each of which transmits signals to a unique set of target proteins. While invertebrates have only one alpha subunit in the G12 class, mammals possess two alpha subunits, Gα12 and Gα13, whose roles include stimulation of cell growth, embryonic development, cytoskeletal changes, and cell migration[3,4]. Regulation of growth and migration by the G12 class of alpha subunits has considerable pathological significance, as overexpressed or mutationally activated forms of Gα12 and Gα13 have been shown to drive cellular oncogenic transformation and metastatic invasion [3,4,7].

Although Gα 12 and Gα 13 share 67% amino acid identity, sequence divergence has allowed these two proteins to develop distinct sets of binding partners[2] (Figure 1). Gα 12 and Gα 13 both bind and activate Rho-specific guanine nucleotide exchange factors (RhoGEFs) like PDZ-RhoGef and leukemia-associated RhoGEF (LARG)[4]. However, Gα 12 interacts with heat-shock protein 90 (Hsp90) and Axin while Gα 13 lacks ability to bind these proteins[4]. Identification of these non-redundant sets of binding partners has sparked further investigation of the ways by which Gα 12 and Gα 13 have diverged in their mechanisms of growth signaling. Activation of the serum response element (SRE) pathway is one mechanism by which Gα 12 and Gα 13 regulate cellular growth and oncogenic transformation[6,7]. Activation of RhoGEFs by both Gα 12 and Gα 13 and downstream activation of protein RhoA facilitates the nuclear translocation of myocardin-related transcription factor (MRTF-A), a transcriptional co-activator of the serum response factor (SRF)[5,6,8,9]. Activated SRF binds to the serum response element (SRE) and leads to the transcription of early response growth genes such as the proto-oncogene *c-fos* (Figure 1)[5,6,8,9]. While both proteins robustly signal through the SRE pathway through RhoGEFs, increasing evidence suggests that Gα 12 and Gα 13 drive this growth response via non-redundant methods. Montgomery et al. (2014) found that inhibition of Hsp90, a Gα 12-specific effector, lowers SRE signaling to a greater extent in Gα 12 than in Gα 13[10]. Also, Gα 12 and Gα 13 harbor several regions of divergent amino acid sequence, such as the N-terminal domain and a C-terminal variable region. This latter region was found to be necessary for SRE signaling in Gα 12 but not Gα 13[10]. These results indicate that Gα 12 and Gα 13 have evolved different structural and functional requirements for growth signaling. I hypothesized that sequence divergence between Gα 12 and Gα 13 in the C-terminal region allowed Gα 12 to acquire a unique set of binding partners. In order to investigate the role of the variable region in Gα 12-specific effector binding, chimeric Gα 13 proteins containing primary sequence from Gα 12 in multiple regions, including the C-terminal variable region, were constructed using PCR-based mutagenesis. Protein co-precipitation experiments using these chimeric constructs showed that substitution of the variable region of Gα 13 with sequence from Gα 12 was sufficient to bestow Gα13 with the ability to bind Gα12-specific effector proteins such as Hsp90, Polycystin-1, and the serine/threonine-protein kinase ARAF. As certain cancers only overexpress one of the two G12 alpha subunits, our characterization of this variable region in Gα12 can be used to guide the development of drugs that inhibit Gα12-mediated growth signaling[11,12].

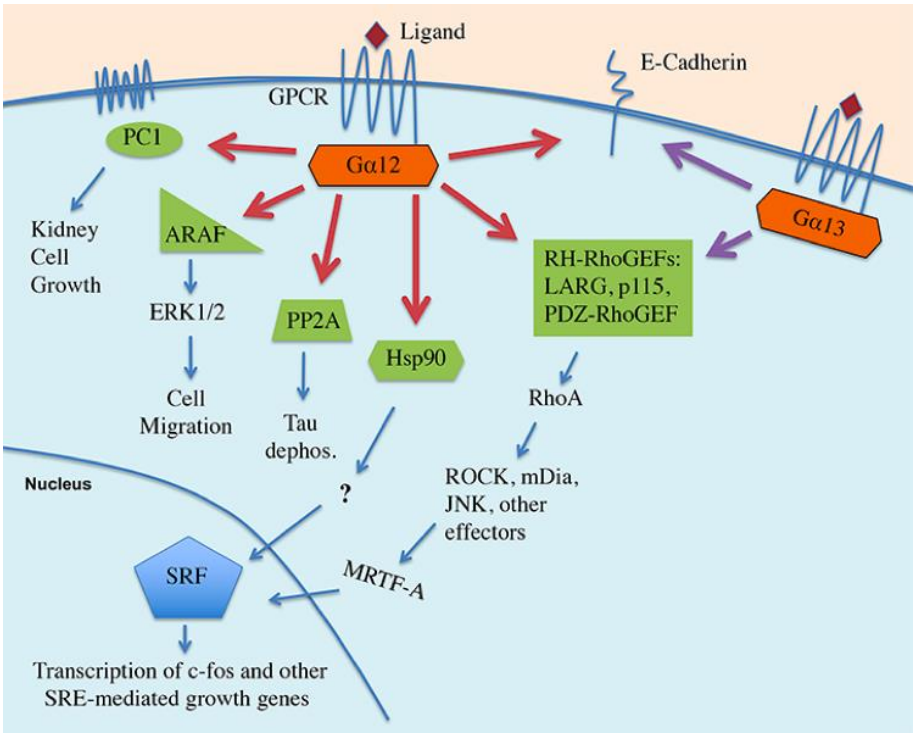

Figure 1. Selected signaling targets and cellular responses mediated by the G12/13 subfamily of trimeric G proteins.

Figure 1. Upon activation by GPCR, Gα12 and Gα13 regulate various cellular responses by activating a wide range of effector proteins. Gα12/13 regulate growth signaling through Rho-dependent nuclear translocation of MRTF-A, a transcriptional co-activator that allows SRF to bind the SRE promoter and induce transcription of early growth response genes like c-fos. Selected binding partners of both Gα12 and Gα13 are shown, including rgs-homology (RH)-RhoGEFs and E-cadherin, as well as Gα12-specific targets such as ARAF, Hsp90, polycystin-1 (*PC1*), and protein phosphatase-2A (*PP2A*).

2. Materials and Methods

2.1 DNA Constructs

The serum response element (SRE) luciferase plasmid was a gift from Channing Der (University of North Carolina, Chapel Hill). All point mutants and chimeric variants of Gα12 and Gα13 were engineered using PCR-based mutagenesis. Each construct began with two or three initial PCR amplimers, derived from Gα12 or Gα13, designed to have 19-20 bp overlap with the adjacent amplimer (Figure 2). Templates were Gα12 and Gα13 cDNAs encode myc-tagged, activated variants (glutamine to leucine mutation) of the alpha subunit. Primary PCR products were gel-extracted and "sewn" together in a secondary round of PCR using end primers containing 5'-end restriction sites for cloning into the mammalian expression plasmid pcDNA3.1 (Figure 2). All mutant plasmid constructs were purified and then verified by sequencing (Genewiz, South Plainfield, NJ).

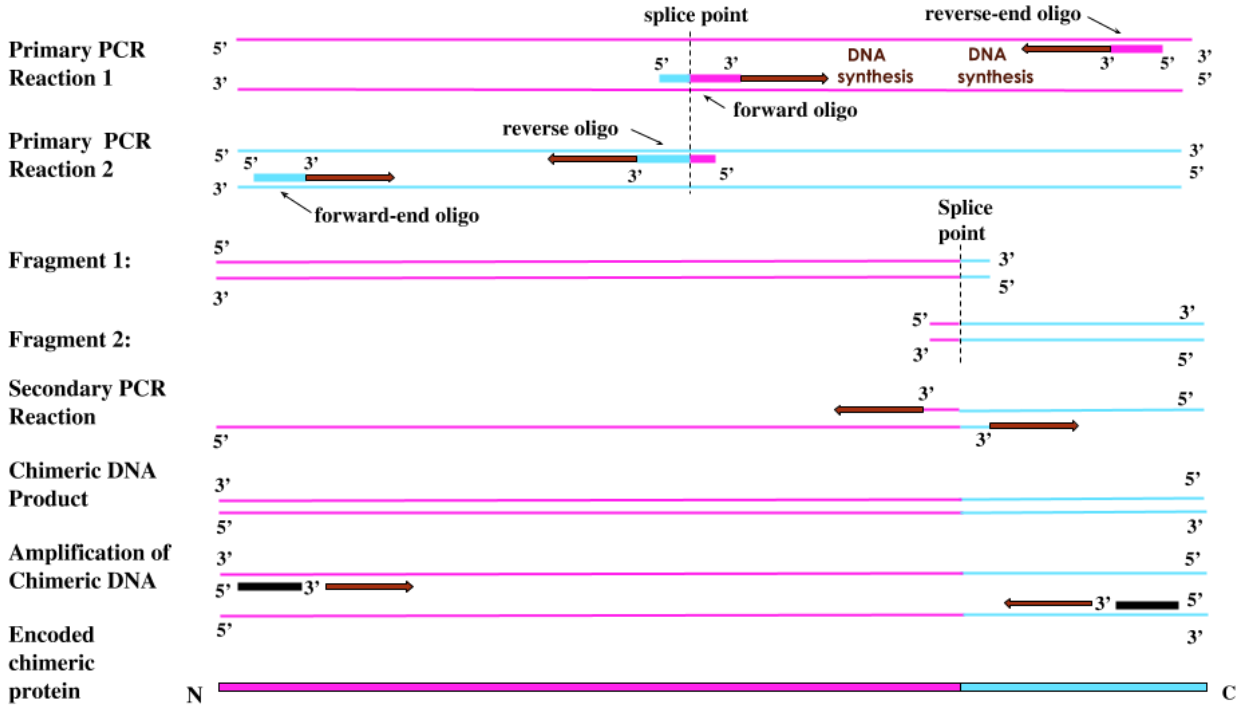

Figure 2. Chimeric Protein Construction.

Figure 2. PCR-based mutagenesis was used to create an array of chimeric proteins containing amino acid sequence from both Gα12 and Gα13. Specific internal oligonucleotides were used in primary PCR reactions with either Gα12 and Gα13 template DNA to create two or three amplimers with 19-21 base pairs of overlap. These amplimers were combined in a secondary PCR reaction to create a final chimeric DNA product that was molecularly cloned into a mammalian expression plasmid.

2.2 Reporter Gene Assays

HEK293 cells grown to approximately 80% confluence in 12-well plates were transfected with 0.2 mg of SRE luciferase, 0.02 mg of pRL-TK harboring the cDNA for *Renilla* luciferase (Promega), and 50 ng of plasmid encoding myc-Gα12-QL, myc-Gα13-QL, or a chimeric Gα12-QL or Gα13-QL variant. Cells were transfected using polyethylenimine (PEI; 3 µg per sample) and luminometry assays were performed ~48 hours post-transfection. Each well was washed with 1 mL of 1X PBS, lysed with 250 µL of 1X passive lysis buffer (Promega), and agitated 20 minutes at 120 rpm. We analyzed lysates using a Dual-luciferase assay system and GloMax 20/20 luminometer (Promega, Madison, Wisconsin). Light output from firefly luciferase activity was divided by *Renilla* luciferase activity to normalize for variations in transfection efficiency. SDS-PAGE and immunoblotting using anti-Gα12 (Santa Cruz Biotechnology, Dallas, Texas), anti-Gα13 (Millipore, Madison, Wisconsin) and anti-myc (Millipore) epitope antibodies were used to monitor levels of G protein expression in each sample.

2.3 Preparation of Detergent-Soluble Proteins

Human embryonic kidney cells (HEK293) were grown in Dulbecco's modified Eagle's medium (Corning, Corning, New York) supplemented with 10% fetal bovine serum (Gibco, Waltham, MA). We used PEI to transfect a 10-cm dish of 90% confluent HEK293 cells with 7 µg of plasmid DNA encoding G protein variants. Cells were PBS-washed and scraped from the dish 32–40 h post-transfection, then centrifuged 500 x *g* for three minutes. Pellets were resuspended and solubilized in 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 mM), pepstatin (1.45 mM), Na-tosyl-L-lysine chloromethyl ketone (58 mM), tosyl-L-phenylalanylchloromethane (61 mM), and phenylmethylsulfonyl fluoride (267 mM). Lysates were continuously inverted at 4°C for 30 minutes and centrifuged at 80,000 x *g* for one hour. Supernatants were snap-frozen and stored at -80°C.

2.4 Protein Interaction Assays

Cell lysate extracts from transfected HEK293 cells were diluted ~18-fold in HEDM buffer [50 mM HEPES pH 7.5, 1 mM EDTA, 3 mM dithiothreitol, 10 mM $MgSO_4$] to decrease the detergent concentration. We reserved 3% of each diluted lysate sample prior to the interaction experiment for later analysis. Sepharose-bound GST-fusion proteins were diluted by ~10-fold with HEDM buffer and added to the lysate samples. The resulting mixture was inverted continuously for ~2 hours at 4°C. Samples were centrifuged at 1,300 x *g* and washed twice with HEDM buffer containing 0.05% polyoxyethylene-10-lauryl ether. Pelleted samples were subject to SDS-PAGE and immunoblotting using a primary antibody for Gα12 or Gα13 and a secondary antibody (Promega) conjugated with alkaline phosphatase. BCIP and NBT were used for development.

3. Results

3.1 Successful Chimeric Protein Construction and Antibody Detection

PCR-based mutagenesis was used to create an initial set of fourteen chimeric proteins (Figure 3). Each chimeric construct harbors a myc epitope and an activating Glutamine to Leucine (QL) mutation that abolishes intrinsic GTPase activity of the alpha subunit. As each chimera contains a unique arrangement of amino acid sequence from both Gα12 and Gα13 in addition to an internal myc epitope, a trifold antibody screening was used to determine which antibody should be used for optimal detection of each construct. Protein lysate from a select eight chimeric constructs was subject to immunoblotting analysis using three different primary antibodies: anti-Gα13, anti-Gα12, and anti-myc epitope. Chimeras 201, 304, and 306 were strongly detected by anti-Gα12, while chimeras 106, 204, 206, 207, and 306 were strongly detected by the anti-Gα13 (Figure 4). Chimeras 106, 204, 206, 207, 304, 306 were also detected by anti-myc. Chimera 305 was undetected by all three antibodies (Figure 4).

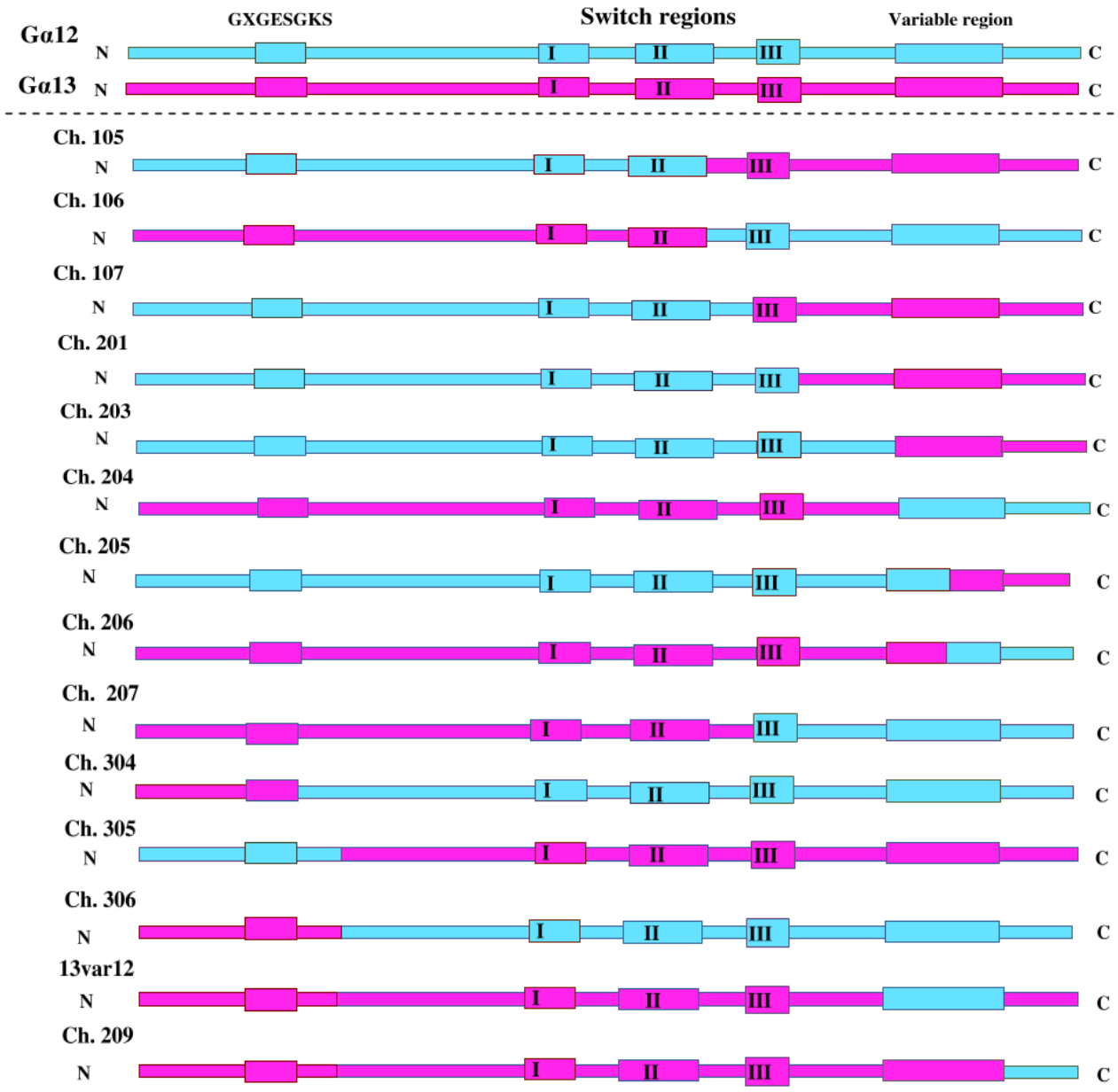

Figure 3. Schematic of chimeric Gα12 and Gα13 proteins.

Figure 3. Fourteen unique chimeric constructs were engineered and harbor sequence from both Gα12 and Gα13 in specific regions. All encoded proteins contain an activating Glutamine to Leucine (QL) mutation, which abolishes GTPase activity, and a myc epitope tag.

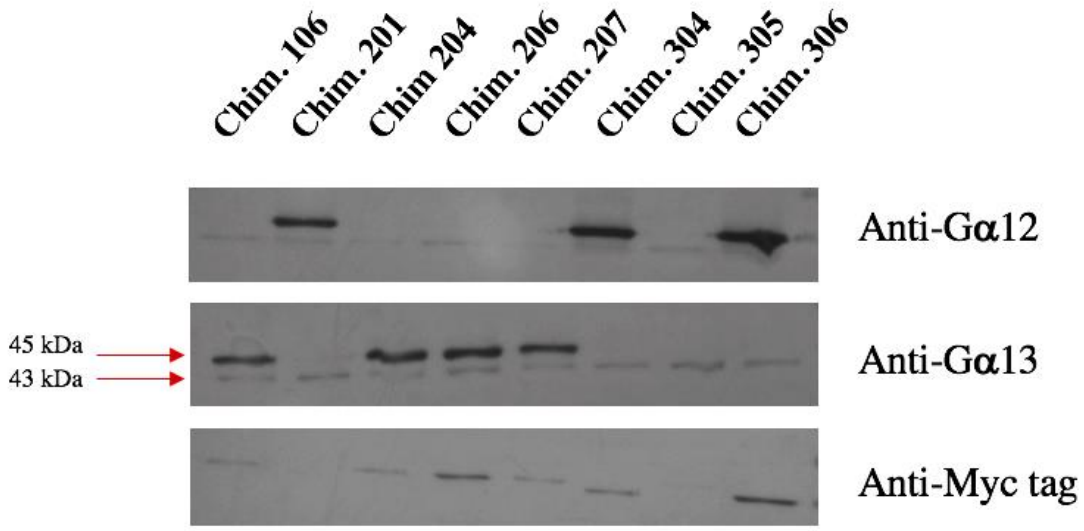

Figure 4. Antibody screening for detection of selected chimeras.

Figure 4. Eight select Gα12 and Gα13 chimeras were subject to immunoblotting analysis with three different antibodies: anti-Gα12, anti-Gα13, and anti-myc epitope. All chimeric constructs appear as the upper band (45 kDa), and genomic Gα13 and Gα12 appear as the lower band (43 kDa) on the anti-Gα12 and anti-Gα13 blots.

3.1 Select C-Terminal Chimeras Retain SRE-Mediated Growth Signaling

Luminometry assays were used to quantify the ability of a select three C-terminal Gα13 chimeras to engage in SRE-mediated growth signaling. Chimeric plasmids were co-transfected into HEK293 cells with a SRE-dependent firefly luciferase reporter plasmid and a G protein-independent *Renilla* luciferase reporter plasmid. Firefly luciferase activity was divided by *Renilla* luciferase activity to account for variations in transfection efficiency, and each ratio was displayed as a percentage of the positive control (Gα13-QL) to account for inter-experimental variability. Gα12-QL had an average Firefly/*Renilla* ratio at 88% of Gα13-QL, and all chimeras had an average Firefly/*Renilla* luminescence ratio within 73-90% of Gα13-QL (Figure 5). Each cell lysate sample was subject to immunoblotting to monitor differences in protein expression, and all chimeric constructs as well as Gα13 showed robust expression (Figure 5). The lighter bands observed for Gα12-QL are likely due to less efficient antibody detection instead of weak protein expression, as laboratory data has consistently shown that the myc antibody detects Gα13-QL to a greater extent than Gα12-QL (Figure 5). If Gα12 expression was indeed low throughout all six SRE experiments, it is unlikely that the robust SRE signaling for this protein displayed in Figure 5 would have been observed.

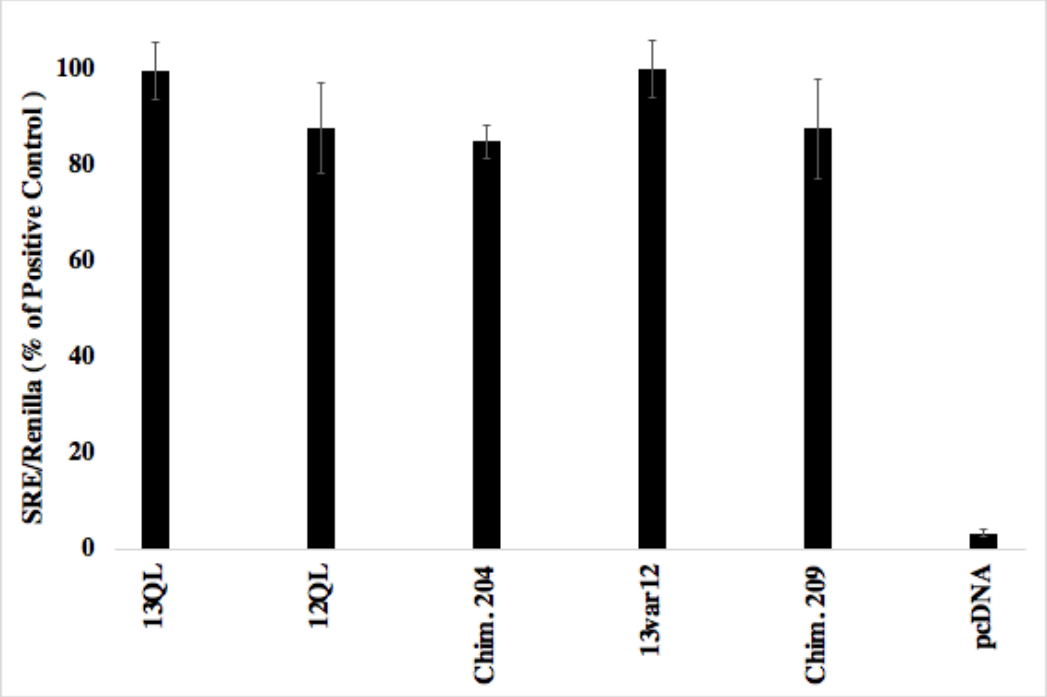

Figure 5. Gα13 chimeras retain SRE-mediated growth signaling function.

Figure 5. Chimeric constructs were assembled as shown in Figure 2 and co-transfected into HEK293 cells along with SRE-Luciferase and *Renilla* reporter plasmids. Plasmid vector pcDNA3.1 was transfected as a negative control. Data presented here are ratios for SRE promoter-dependent luciferase activity (firefly) normalized for G protein-independent thymidine kinase luciferase activity (*Renilla*). Firefly/*Renilla* luminescence ratios for each sample are represented graphically as an average percent of the positive control (Gα13-QL) for six replicates. Gα13QL-and Gα12-QL both contain internal myc tags. Error bars represent the range among the replicates from the average percent of the positive control. Protein expression was verified by immunoblotting with an anti-myc antibody as shown in a representative blot.

3.2 Variable Region Bestows Gα13 with Gα12-Specific Binding

Protein interaction experiments were performed using select C-terminal Gα13 chimeras and a variety of GST-fusion Gα12-specific target proteins. Cell lysate from untransfected cells was used as a negative binding control and showed no interaction with any of the effectors (Figure 6b). Gα13-QL was included in each experiment and did not show strong interaction with any of the Ga12-specific effector proteins (Figure 6b). The Gα12/13 effector LARG was used as a positive binding control and showed robust interaction with Gα13-QL and all chimeric constructs (Figure 6b). Chimera 106 contains Gα12 sequence from the end of the switch two region through the C terminus of Gα13 (Figure 6a) and showed strong interaction with the Gα12-specific effectors ARAF, Axin, and A-kinase anchor protein (AKAP; Figure 6b). Chimera 204 has sequence from Gα12 at the 42-residue variable region through the C terminus of Gα13 (Figure 6a) and also showed strong interaction with the latter Gα12-specific target proteins (ARAF, Axin, and AKAP; Figure 6b). Chimera 204 was subdivided to make chimeras 13var12 and 209. Chimera 13var12 contains Gα12 sequence solely at the variable region (Figure 6a) and displayed robust interaction with a variety of Gα12 specific effector proteins (Hsp90, PC1, ARAF, and the scaffolding Aα subunit of PP2A; Figure 6b). Chimera 209 contains Gα12 sequence only at the C-terminal region of Gα13 (Figure 6a) and lacked the ability to bind to Gα12-specific targets (Figure 6b). These binding results indicate that the variable region of Gα12 is sufficient to bestow Gα13 with the ability to interact with various Gα12-specific effector proteins.

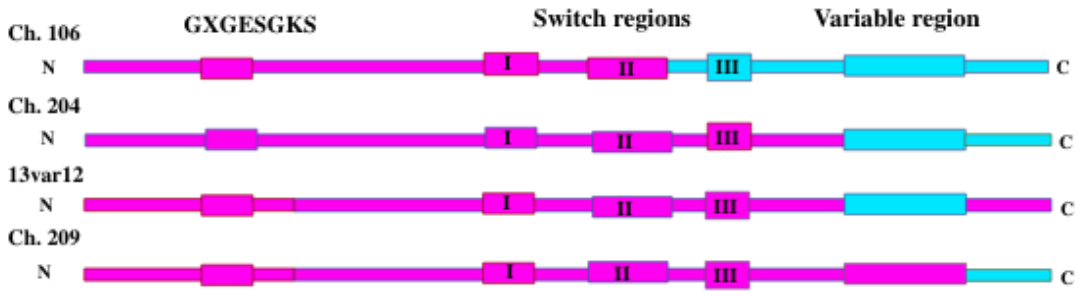

Figure 6a. Schematic of C-terminal chimeric Gα13 proteins.

Figure 6a. The above chimeric proteins were engineered to have primary sequence from Gα12 (blue) at C-terminal regions of interest in Gα13 (pink). All chimeras contain an activating Glutamine to Leucine (QL) mutation and an internal myc epitope.

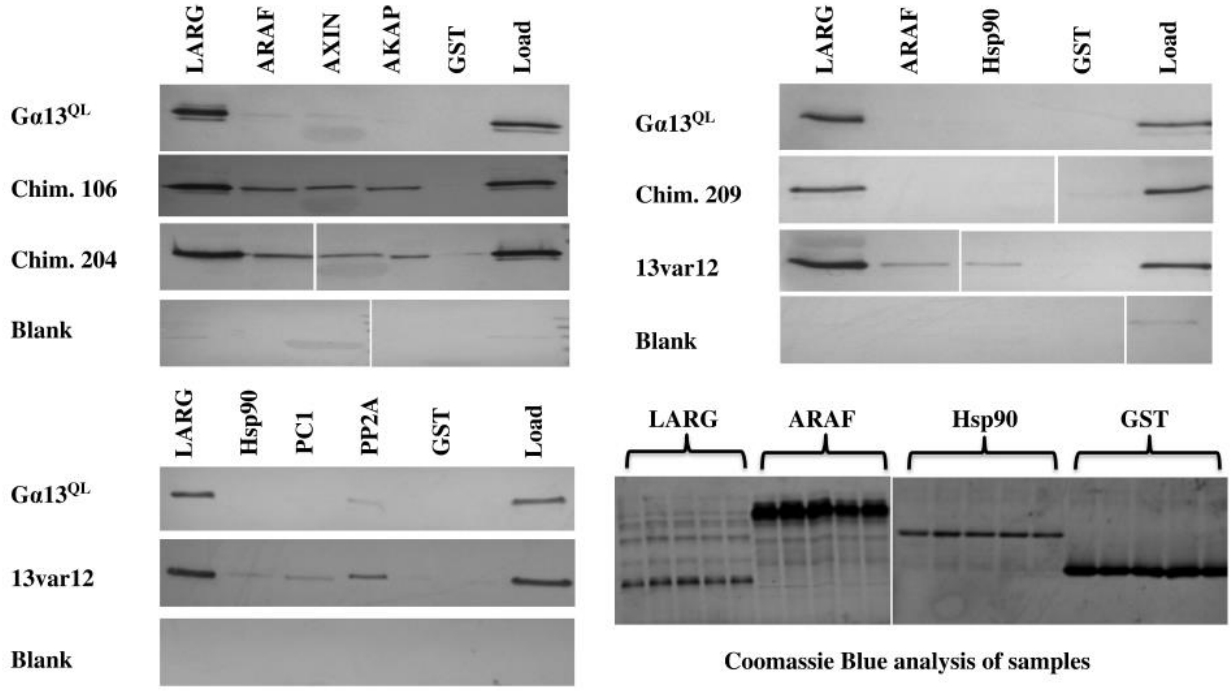

Figure 6b. The Variable region of Gα12 harbors determinants for binding multiple Gα12-specific targets.

Figure 6b. Protein co-precipitation experiments were performed using C-terminal Gα13 chimeric lysates and a variety of GST-fusion Gα12-specific target proteins. A blank lysate from untransfected cells was used as a negative binding control. Uniformity of GST-fusion protein levels in different samples are shown for a representative experiment (bottom right panel).

4. Conclusions

The G12/13 class is the only subfamily of heterotrimeric G proteins capable of transforming fibroblasts through wild-type overexpression[7]. This unique oncogenic property has incited extensive investigation of the structural regions of Gα12 and Gα13 involved in growth signaling through the SRE pathway. Although both Gα12 and Gα13 stimulate the

SRE pathway in RhoA-dependent manners, research has shown that these two alpha subunits have distinct mechanisms for SRE-mediated growth signaling[4,7,10,14]. As Gα12 and Gα13 have diverged in sequence following evolutionary G12 gene duplication, it is likely that their unique growth signaling mechanisms are a result of differential interactions with effector proteins in the cell. X-ray crystallographic analysis of Gα12 in complex with effector proteins has proven challenging for many researchers, as this method requires a copious amount of functional, homogenized Gα12[13]. In lieu of a crystal structure, mutagenic approaches have proven to be viable methods for identifying regions of Gα12 that interact with specific effector proteins.

Previous laboratory data has shown that a variable 42-residue region is necessary for SRE-mediated growth signaling in Gα12 but not Gα13[10]. However, the functional role of this region in relation to effector binding has not yet been investigated. This project used a mutagenic approach to define the functional properties of variable N and C terminal regions in Gα12 target protein binding, with a particular focus on the variable 42-residue C-terminal region. Select C-terminal chimeras 204, 13var12, and 209 were then tested for their ability to stimulate the SRE growth pathway, as robust stimulation of the SRE growth pathway serves as a proxy for correct protein folding and functioning within the cell. All three chimeras retained their ability to drive SRE-mediated growth signaling, suggesting that these three constructs are able to properly fold and interact with effector proteins in the cell. Protein co-precipitation experiments revealed that the C-terminal Gα13 chimera 204 gained the ability to interact with Gα12-specific effectors including AKAP, Axin, and ARAF. Chimera 204 was subdivided into chimeras 13var12 and 209 in order to assess the role of the variable region in Chimera 204's gain-of-function binding. Chimera 13var12 showed strong interaction with ARAF, Hsp90, PC1, and and the scaffolding Aα subunit of PP2A. Chimera 209 did not show any interaction with the latter target proteins, suggesting that the variable region of Gα12 was able to bestow Gα13 with the ability to bind Gα12-specific effectors.

Montgomery et al. (2014) previously reported that the variable 42-residue region was a determinant for SRE-mediated growth signaling in Gα12 but not Gα13[10]. The protein interaction results in this study suggest that this C-terminal region is also involved in Gα12-specific effector binding with target proteins such as Hsp90. The variable region's role in Gα12-Hsp90 interaction may also contribute to Gα12-specific mechanisms of growth signaling, which is supported by the Vaiskunaite et al. (2014) finding that Hsp90 interaction was required for Ga12-induced SRE activation[14]. Thus, divergence between Gα12 and Gα13 in this variable 42-residue region may have helped Gα12 develop unique effector interactions that contribute to distinct mechanisms for its SRE-mediated growth signaling.

Although both Gα12 and Gα13 have significant roles in oncogenic transformation and cancer metastasis, certain types of cancer types preferentially overexpress one of the two G12 alpha subunits[7,12,13]. For example, a particular chemokine receptor-Gα13 signaling axis has been shown to drive metastatic breast cancer migration, while a Gα12-RhoA signaling axis has been shown to stimulate oral cancer metastasis[12,13]. With the non-redundant oncogenic roles of Gα12 and Gα13, potential therapies for Gα12 driven cancers may need to target subunit-specific effector interactions in order to disturb Gα12-driven cell growth and tumor invasion. Thus, further characterization of the 42-residue variable region in Gα12 may help guide the development of inhibitors that can be used disrupt the various Gα12-specific effector interactions that contribute to Gα12's oncogenic activity.

5. Acknowledgments

The author would like to acknowledge support from the GlaxoSmithKline Foundation, Lineberger Comprehensive Cancer Center, and UNC-Asheville Undergraduate Research Program. I would also like to thank Dr. Ted Meigs, Dr. Nicole Dover, Makenzy Mull, and the rest of my wonderful laboratory for their constant support throughout my project.

6. References

1. Oldham, William M., and Heidi E. Hamm. "Heterotrimeric G Protein Activation by G-Protein-Coupled Receptors." *Nature Reviews Molecular Cell Biology* 9 (January 1, 2008): 60.

2. Strathmann, M P, and M I Simon. "G Alpha 12 and G Alpha 13 Subunits Define a Fourth Class of G Protein Alpha Subunits." *Proceedings of the National Academy of Sciences of the United States of America* 88, no. 13 (July 1, 1991): 5582–86.

3. Suzuki, Nobuchika, Nicole Hajicek, and Tohru Kozasa. “Regulation and Physiological Functions of G12/13-Mediated Signaling Pathways.” *Neuro-Signals* 17, no. 1 (February 2009): 55–70. https://doi.org/10.1159/000186690.

4. Kelly, Patrick, Patrick J. Casey, and Thomas E. Meigs. “Biologic Functions of the G12 Subfamily of Heterotrimeric G Proteins: Growth, Migration, and Metastasis.” *Biochemistry* 46, no. 23 (June 2007): 6677–87. https://doi.org/10.1021/bi700235f.

5. Yu, O. M., and J. H. Brown. “G Protein-Coupled Receptor and RhoA-Stimulated Transcriptional Responses: Links to Inflammation, Differentiation, and Cell Proliferation.” *Molecular Pharmacology* 88, no. 1 (June 9, 2015): 171–80. https://doi.org/10.1124/mol.115.097857.

6. Fromm, Christian, Omar A Coso, Silvia Montaner, Ningzhi Xu, and J Silvio Gutkind. “The Small GTP-Binding Protein Rho Links G Protein-Coupled Receptors and Gα(12) to the Serum Response Element and to Cellular Transformation.” *Proceedings of the National Academy of Sciences of the United States of America* 94, no. 19 (September 16, 1997): 10098–103.

7. Juneja, Juhi, and Patrick J Casey. “Role of G12 Proteins in Oncogenesis and Metastasis.” *British Journal of Pharmacology* 158, no. 1 (September 2009): 32–40. https://doi.org/10.1111/j.1476-5381.2009.00180.x.

8. Siehler, Sandra. “Regulation of RhoGEF Proteins by $G_{12/13}$ -Coupled Receptors.” *British Journal of Pharmacology* 158, no. 1 (September 2009): 41–49. https://doi.org/10.1111/j.1476-5381.2009.00121.x.

9. Hill, Caroline S., Judy Wynne, and Richard Treisman. “The Rho Family GTPases RhoA, Racl, and CDC42Hs regulate Transcriptional Activation by SRF.” *Cell* 81, no. 7 (June 1995): 1159–70. https://doi.org/10.1016/S0092-8674(05)80020-0.

10. Montgomery, E. R., B. R. S. Temple, K. A. Peters, C. E. Tolbert, B. K. Booker, J. W. Martin, T. P. Hamilton, et al. “G 12 Structural Determinants of Hsp90 Interaction Are Necessary for Serum Response Element-Mediated Transcriptional Activation.” *Molecular Pharmacology* 85, no. 4 (February 21, 2014): 586–97. https://doi.org/10.1124/mol.113.088443

11. Gan, Chai Phei et al. “Heterotrimeric G-protein alpha-12 (Gα12) subunit promotes oral cancer metastasis” *Oncotarget* vol. 5,20 (2014): 9626-40.

12. Yagi, Hiroshi et al. “A synthetic biology approach reveals a CXCR4-G13-Rho signaling axis driving transendothelial migration of metastatic breast cancer cells” *Science signaling* vol. 4,191 (2011): ra60.

13. Kreutz, Barry et al. “A new approach to producing functional G alpha subunits yields the activated and deactivated structures of G alpha(12/13) proteins” *Biochemistry* vol. 45,1 (2006): 167-74.

14. Vaiskunaite, Rita, et al. “Interaction between the Gα Subunit of Heterotrimeric G12Protein and Hsp90 Is Required for Gα12Signaling.” *Journal of Biological Chemistry*, vol. 276, no. 49, Nov. 2001, pp. 46088–46093., doi:10.1074/jbc.m108711200.