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Mutational Analysis of a Conserved C-terminal Region in Gα12 and Gα13 Harbors Unique Determinants of SRF Signaling and RhoGEF Binding

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Abstract

Heterotrimeric guanine-nucleotide binding proteins (G proteins) of the G12/13 subfamily play key signaling roles in cell growth, oncogenic transformation, migration, and invasion. In this subfamily, mammals encode two distinct G protein alpha subunits (Gα12 and Gα13) which share 67% amino acid identity. Gα12/13 also share several downstream signaling pathways, including transcriptional activation via Serum Response Factor (SRF). However, both proteins have multiple unique binding partners, suggesting divergent signaling mechanisms within this subfamily. Amino acid substitutions from non-SRF signaling invertebrate homologs were utilized to identify different structural features required for growth signaling in Gα12 and Gα13. Previous work showed that replacing the N-terminal region of these mammalian α subunits with invertebrate amino acid sequence caused no disruption of SRF signaling. Conversely, invertebrate substitution of a highly conserved sequence downstream of the Switch III region abolished SRF signaling by Gα12 and Gα13. Current studies of this conserved "Post-Switch" region of Gα12 showed a single amino acid substitution disrupted signaling to SRF, while the same mutation in Gα13 had no effect. Replacement of additional amino acids in this region were required to uncouple Gα13 from SRF activation, suggesting divergent growth signaling mechanisms in the G12/13 subfamily. Additionally, invertebrate sequence substitution in the region disrupted Gα13 interaction with cancer-implicated proteins, termed RhoGEFs, whereas the corresponding SRF-uncoupled Gα12 Post-Switch mutant retained binding to these effector proteins. These findings revealed novel C-terminal motifs in Gα12 and Gα13 that are necessary for their non-overlapping mechanisms of RhoGEF binding and SRF-mediated cell growth. Since certain cancers selectively overexpress Gα12 or Gα13, further characterization of these proteins could be used in the development of Gα12-Gα13-specific inhibitors.

1. Introduction

Cells have a variety of pathways capable of transducing extracellular information into diverse intracellular responses. These signaling pathways are typically initiated by a ligand binding and activating a membrane-bound receptor. G protein-coupled receptors (GPCRs) are integral membrane proteins that respond to a variety of ligands including hormones, neurotransmitters, and olfactory molecules [1]. GPCRs transmit signals to a heterotrimeric G protein tethered to the cytoplasmic surface of the membrane, which consists of an α subunit and a β-ɣ dimer. After GPCR activation, the alpha subunit releases GDP and enters an activated, GTP-bound state, separated from the beta and gamma subunits. The GTP-bound alpha subunit is capable of interacting with downstream effector proteins, leading to cell growth and cytoskeletal changes. Four subfamilies of alpha subunits, Gs, Gi, Gq, and G12, transmit signals to unique sets of target proteins.

The mammalian G12/13 subfamily evolved from a duplication in invertebrates, which possess a single alpha subunit. Gpa-12 is the invertebrate G12/13 subfamily homolog from roundworms, and it is involved in cell growth and signaling. Mammalian Gα12 and Gα13 also stimulate cell growth, embryonic development, cytoskeletal changes, and cell migration [1]. Due to the similarities in amino acid sequence and downstream proteins, like CeRho-GEF, Gpa-

12 is an ideal candidate for mutational analysis to better understand the structural differences that evolved in mammalian Gα12 and Gα13 [2]. Gα12 and Gα13 still share 67% amino acid identity, which allows them to use many of the same downstream binding partners (Figure 1). Both proteins bind and activate Rho-specific guanine nucleotide exchange factors with an RGS homology domain (RH-RhoGEFs), including p115, PDZ-RhoGEF, and leukemia-associated RhoGEF (LARG) [3]. Activation of the serum response element (SRE) pathway is one mechanism by which Gα12/13 regulate cell growth and oncogenic transformation [4]. Activation of RH-RhoGEFs by Gα12/13 activates RhoA, stimulating the nuclear translocation of myocardin-related transcription factor (MRTF-A). MRTF-A is a transcriptional co-activator of the serum response factor (SRF), and activated SRF binds to the serum response element (SRE), leading to transcription of early growth genes like the proto-oncogene c-fos (Figure 1) [5,6].

While both Gα12 and Gα13 strongly signal through the SRE pathway via RH-RhoGEFs, increasing evidence suggests that these proteins use differing methods to drive this growth response . A portion of the C-terminal region is divergent between Gα12 and Gα13, and the region has been shown to be necessary for SRE signaling in Gα12 but not Gα13 [1]. Previous research focused on this divergent region, but adjacent to it is a highly conserved region of about thirty-six amino acids, containing only eight amino acids that differ between Gα12/13 and Gpa-12. This conserved region will be referred to as the "Post-Switch" region because of its location directly after the Switch III region and adjacent to the divergent region. In order to investigate the role of the Post-switch region in effector binding, chimeric Gα12 and Gα13 proteins containing sequence from Gpa-12 in the region were constructed using PCR-based mutagenesis. Protein co-precipitation experiments and SRE luminometry assays were performed and results indicated that this conserved region of Gα12/13, and Gpa-12, must play a key role in the structural and functional requirements of growth signaling.

Regulation of cell growth and migration has pathological significance, as overexpressed or mutationally activated forms of Gα12 and Gα13 have been shown to drive oncogenic transformation and metastatic invasion [1,4,7]. Soon after their discovery, it was found that Gα12 and Gα13 are the only two alpha subunits that drive tumorigenesis in their wild-type form due to simple overexpression [4]. Furthermore, Juneja and Casey studied the connection of GPCRs coupled to the G12/13 subfamily with certain cancers, like adenocarcinoma of the breast, prostate, ovary, and lungs [4]. Findings indicated that G12/13 signaling is not only important to tumorigenesis, but also in the early steps of the metastasis process by stimulating tumor cell invasion and entry into the bloodstream [4]. Since certain cancers selectively overexpress Gα12 or Gα13, further characterization of these proteins could be used to guide the development of Gα12 or Gα13-specific inhibitory drugs.

The specific goals of this study were to identify critical residues of Gα13 that are necessary for SRF-mediated signaling and downstream protein interactions with RH-RhoGEFs. The motivation behind the research was previous work with the adjacent Divergent region and Post-switch SRE luminometry data. It was shown by previous students that substitutions of the entire Post-switch region in Gα12 and Gα13 with sequence from Gpa-12 abolished SRF signaling. Point mutations were also made for each of the eight amino acids; two chimeras from Gα12/Gpa-12 suppressed SRE signaling while the analogous point mutations in Gα13/Gpa-12 had no effect. This led to my project to characterize the combination of amino acids necessary for Gα13 growth signaling through SRF and RH-RhoGEF interactions. I also hypothesized that sequence conservation between Gα12/13 and Gpa-12 allowed for the proteins to retain shared binding partners, albeit through potentially different signaling mechanisms.

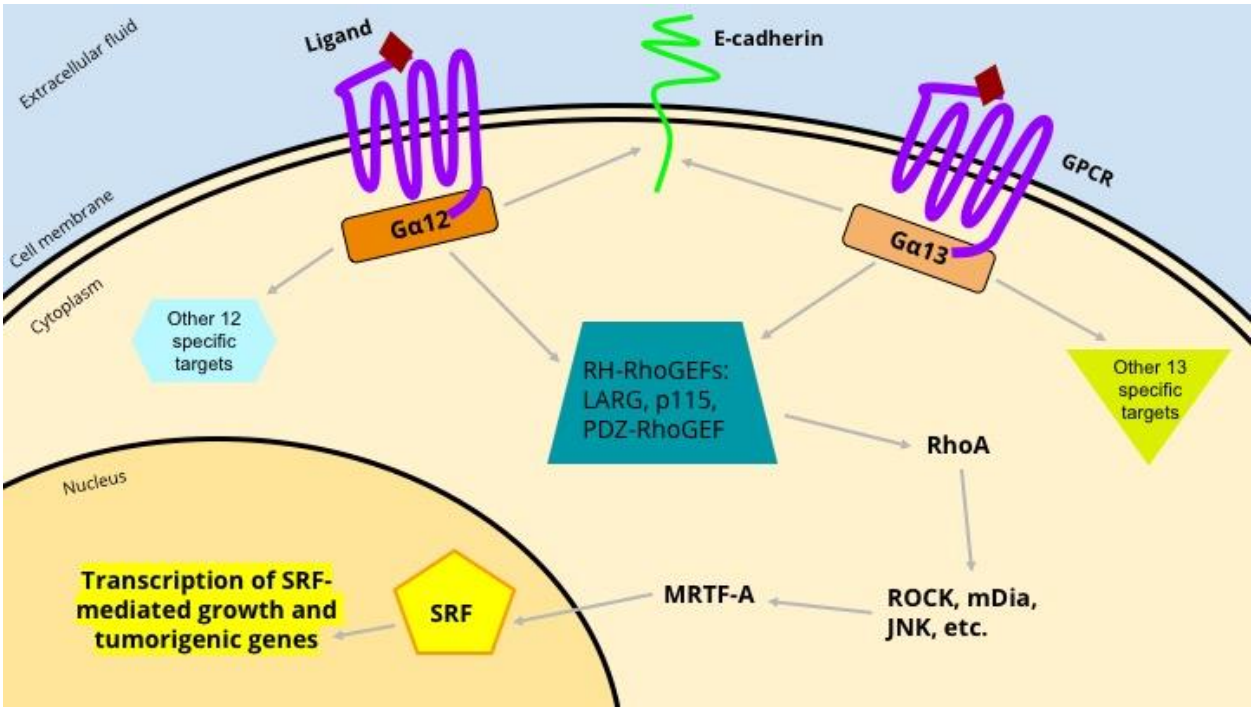

Figure 1. Selected signaling targets and cellular responses mediated by the Gα12/13 subfamily.

Figure 1. After GPCR activation by a specific ligand, Gα12 and Gα13 regulate multiple pathways within the cell, including growth signaling through Rho-dependent nuclear translocation of MRTF-A. This transcriptional co-activator allows SRF to bind the SRE promoter and induce transcription of early growth response genes. Selected binding partners of both Gα12 and Gα13 are shown, including rgs-homology (RH)-RhoGEFs and E-cadherin, though both maintain other specific targets.

2. Methods

2.1 PCR-based Mutagenesis

All variants of the Post-switch region and point mutants of Gα13 and Gpa-12 were engineered using PCR-based mutagenesis. Each construct began with two initial PCR amplimers, derived from Gα13 and the previously constructed Gα13/Gpa-12 Post-switch mutant. They were designed to have 19-20 bp overlap with the adjacent amplimer. The template for Gα13 cDNA encodes a myc-tagged, activated variant (glutamine to leucine mutation) of the alpha subunit. Primary PCR products were gel-extracted and then subjected to a second round of PCR using end primers containing 5'-end restriction sites for cloning into the mammalian expression plasmid pcDNA3.1. All mutant plasmid constructs were purified and then verified by sequencing (Genewiz, NJ).

2.2 Luminometry Assays

Human embryonic kidney cells (HEK293) 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, and 50 ng of plasmid encoding Gα12QL-myc, Gα13QL-myc, or a chimeric Post-switch variant. Cells were transfected using polyethylenimine (PEI), and luminometry assays were performed ~48 hours post-transfection. Transfection with an empty vector (no G protein addition) was used as a control. Each well was washed with 1 mL of 1X PBS, lysed with 250 µL of 1X passive lysis buffer, and cells were disrupted for 20 minutes at 120 rpm. Lysates were evaluated using a dual-luciferase assay system and GloMax 20/20 luminometer (Promega). The light output from firefly luciferase activity was divided by Renilla luciferase activity to control for variations in transfection efficiency. SDS-PAGE and

immunoblotting using anti-Gα12 (Santa Cruz Biotechnology), anti-Gα13 (Millipore), or anti-myc (Millipore) epitope antibodies were used to track levels of G protein expression.

2.3 Preparation of Cell Lysates

HEK293 cells were grown in Dulbecco's modified Eagle's medium (Corning) supplemented with 10% fetal bovine serum (Gibco). We used PEI to transfect a 10-cm dish of 90% confluent HEK293 cells with 10 µg of plasmid DNA encoding G proteins chimeras. Cells were washed with PBS and scraped from the dish ~36 h post-transfection, then centrifuged 500 xg 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 MgSO4, 1% (w/v) polyoxyethylene-10-lauryl ether (LPX)] containing protease inhibitors. Lysates were continuously inverted at 4°C for 30 min and centrifuged at 80,000 xg for one hour. Supernatants were aliquoted and snap-frozen in liquid nitrogen and stored at -80°C.

2.4 Protein Interaction Assays

Cell lysate extracts were diluted in HEDM buffer [50 mM HEPES pH 7.5, 1 mM EDTA, 3 mM dithiothreitol, 10 mM MgSO4] to decrease the detergent concentration. We reserved 3% of each diluted lysate sample prior to the interaction experiment for the positive control. Sepharose-bound GST-fusion proteins were diluted by ~10-fold with HEDM buffer and combined with the lysate samples. The mixtures were inverted continuously on the Orbitron for 90 minutes at 4°C. Samples were kept chilled, centrifuged at 1,300 xg, and washed twice with HEDLM buffer containing 0.05% LPX. Pellets were frequently too small to visualize, so the remaining ~20 µL of sample was mixed with a 1:10 ratio of dithiothreitol (DTT) and 4X protein sample buffer. Samples were subjected to SDS-PAGE and immunoblotting using a primary antibody for Gα12 or Gα13 and a secondary antibody (Promega). Western blots were developed using AP1 buffer, BCIP, and NBT to compare protein binding affinity. Coomassie blue stain was also used as a control to ensure that observed interactions or lack of interactions were not due to GST-fusion protein quantities.

3. Results

3.1 Protein Interaction Assays with Post-switch Mutant Reveal Inhibition of RH-RhoGEF Binding

Previous work in our laboratory revealed that Gα12 and Gα13 harboring an invertebrate Post-switch region failed to drive growth signaling via SRF. We then wanted to test the post-switch mutants' abilities to bind with commonly known downstream binding proteins, like the RH-RhoGEFs. Multiple protein interaction assays were performed using Gα12, Gα13, Gα12 Post-switch, and Gα13 Post-switch with blank cell lysate from HEK293 cells. Interactions were assessed with LARG, p115, PDZ-RhoGEF, and E-cadherin because they are all known to interact with both Gα12 and Gα13. We found the Gα13 Post-switch mutant was inhibited from binding with all RH-RhoGEFs, whereas binding to another G12/13 target protein, E-cadherin, was unaffected (Figure 2). The Gα12 Post-switch mutant's RH-RhoGEF binding was not disturbed, yet both Post-switch mutants failed to activate growth signaling via SRF. All chimeric proteins have an activating glutamine to leucine (QL) mutation and an internal myc epitope tag.

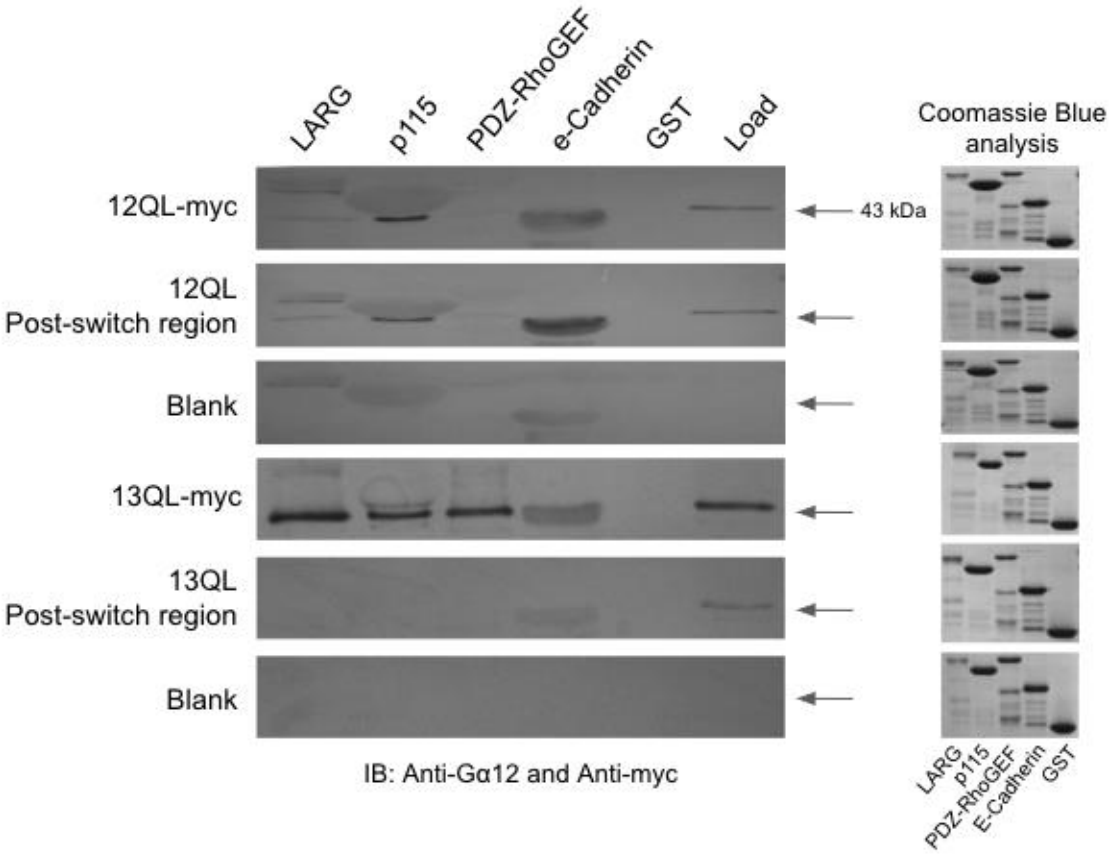

Figure 2. A Post-switch chimera of Gα13 reveals impaired binding of its downstream effector proteins.

Figure 2. Protein co-precipitation experiments were performed using Gα12 and Gα13 Post-switch chimeric lysates and GST-fusion target proteins, RH-RhoGEFs and E-cadherin (*Methods 2.4*). A blank lysate from un-transfected cells was used as a negative binding control. Samples were evaluated using SDS-PAGE and immunoblotting. Uniformity of GST-fusion protein levels in different samples were analyzed using SDS-PAGE and Coomassie blue staining. Grey arrows indicated band size around 43 kDa.

3.2 Successful Creation of Sub-Post-switch Chimeric Proteins

To further dissect the Post-switch region and assess the amino acids responsible for the loss of RH-RhoGEF binding and SRE-mediated signaling, PCR-based mutagenesis was used to create Sub-post-switch mutants. The Post-switch region was divided in two. The first four amino acids that differ between Gα13 and Gpa-12 became the N-terminal Post-switch chimera (N-term Post-switch), while the next four amino acid variants constituted the C-terminal Post-switch chimera (C-term Post-switch). Graphical illustrations of the mutant proteins are depicted in Figure 3, with variable amino acids indicated. The new Sub-post-switch chimeras were subjected to SRE-luminometry and protein binding assays. The N-term Post-switch chimera was unable to stimulate growth signaling via SRF and was unable to interact with RH-RhoGEFs, similar to the Gα13QL Post-switch chimera (Figures 4, 5). Conversely, the C-term Post-switch mutant was uninhibited for both growth signaling and RH-RhoGEF interactions, similar to Gα13QL.

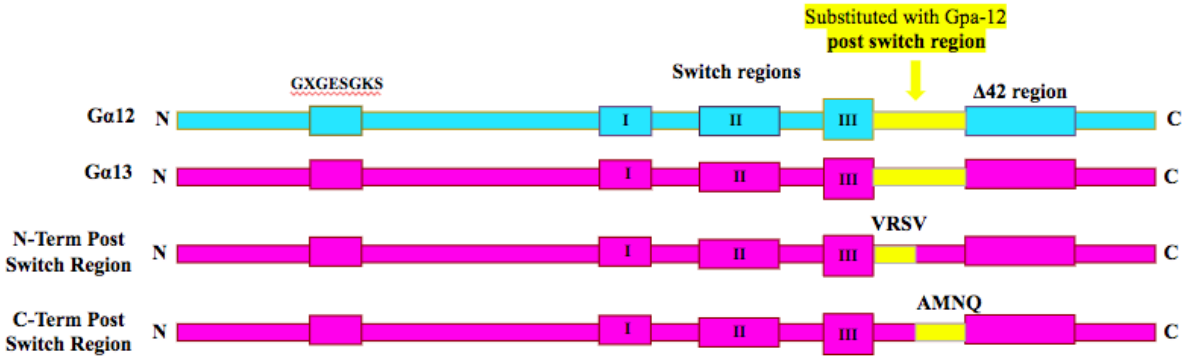

Figure 3. Illustrated representation of invertebrate substitutions in the Post-switch region of Gα12 and Gα13.

Figure 3. The top two Post-switch chimeras of Gα12 and Gα13 were created by a previous student using PCR-based mutagenesis with substitutions of Gpa-12. The abolishment of SRE signaling in the top two chimeric proteins resulted in exploration of protein binding interactions (Figure 2) and the creation of the lower two Post-switch chimeras. The lower two Post-switch chimeras were also created using PCR-based mutagenesis.

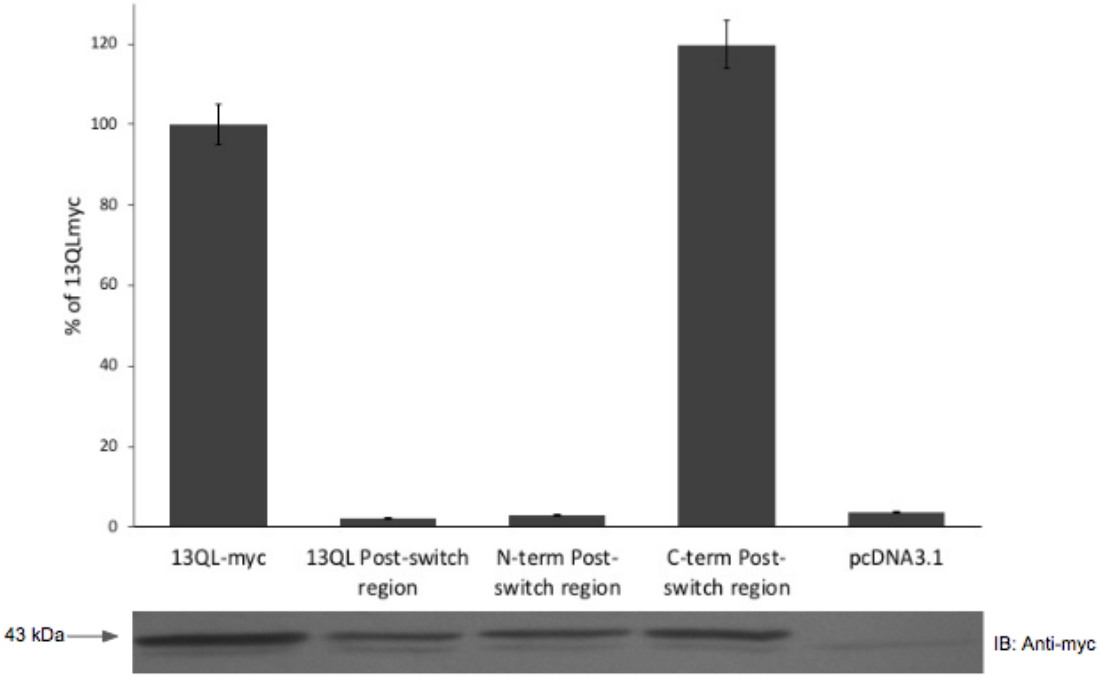

Figure 4. The N-terminal Post-switch chimera fails to signal through SRE-mediated growth.

Figure 4. Chimeric constructs of the Post-switch region were co-transfected into HEK293 cells along with SRE-Luciferase and Renilla reporter plasmids. Plasmid vector pcDNA3.1 was transfected as a negative control.
Data are ratios for SRE promoter-dependent firefly luciferase activity normalized for G protein-independent, thymidine kinase promoter-dependent Renilla luciferase activity. Luminescence ratios for each sample are represented as an average percent of the positive control (Gα13QL) for three replicates. Error bars represent the range among replicates. Protein expression was verified by western blot with an anti-myc antibody (*Methods 2.2*).

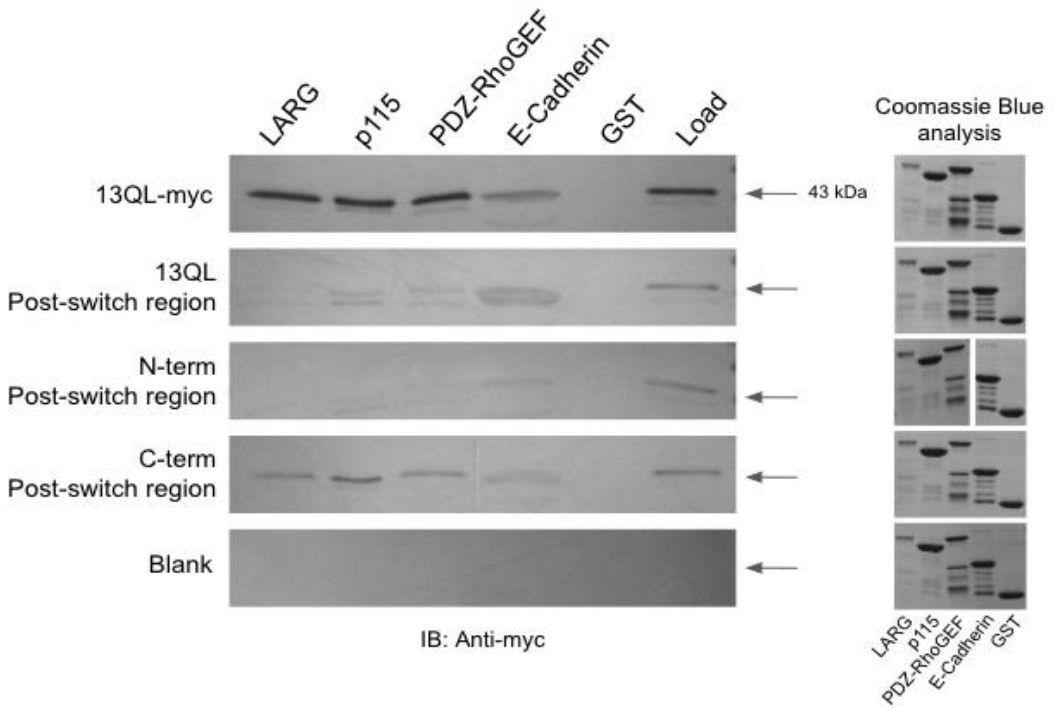

Figure 5. The N-terminal Post-switch chimera of Gα13 inhibits binding with RH-RhoGEFs.

Figure 5. Protein co-precipitation experiments were performed using Gα13 Post-switch chimeric lysates and GST-fused RH-RhoGEFs and E-cadherin (*Methods 2.4*). A blank lysate from untransfected cells was used as the negative binding control. Uniformity of GST-fusion protein levels in different samples were analyzed using SDS-PAGE and Coomassie blue staining. Grey arrows indicated band size around 43 kDa. Similar experimental procedure was conducted for all protein co-precipitation experiments (Figure 2).

3.3 N-terminal Post-switch Point Mutants Development

Novel point mutants were created for individual amino acids within the N-term Post-switch chimera (Figures 6a, 6b). For each mutant, one of the variable amino acids was switched from the Gpa-12 version back to its original Gα13 amino acid using PCR-based mutagenesis (*Methods 2.1*).

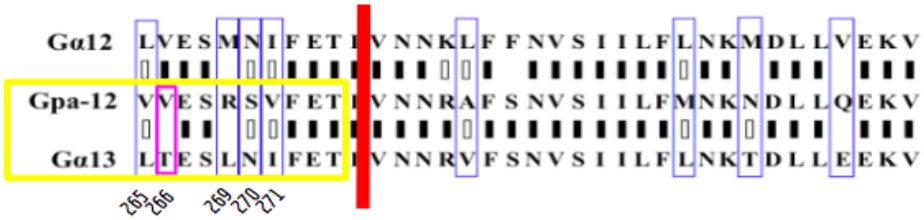

Figure 6a. Aligned Post-switch region sequence of Gα12, Gα13, and Gpa-12.

Figure 6a. Aligned amino acid sequences of Gα12, Gα13, and Gpa-12 within the Post-switch region. Outlined in blue are the 8 amino acids that differ between the three proteins. The red bar represents the divide between the N-term and C-term Post-switch regions. Encircled in yellow is the N-term Post-switch mutant region, with pink outlining the fifth amino acid that differs between only Gα13 and Gpa-12.

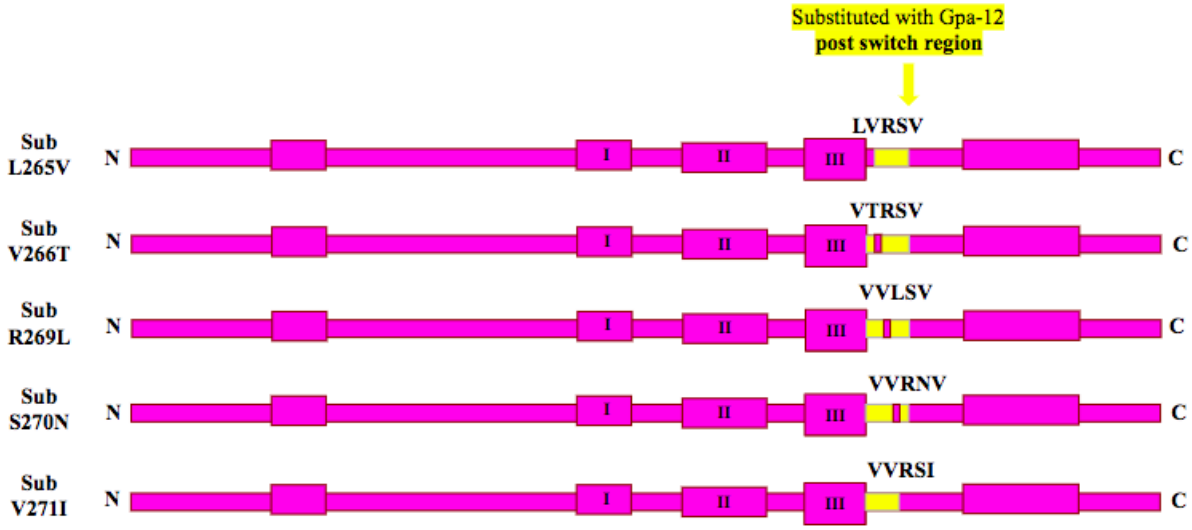

Figure 6b. Illustration of Post-switch mutants within the N-terminal Post-switch region of Gα13.

Figure 6b. Single amino acid substitutions were engineered using PCR-based mutagenesis within the N-term Post-switch chimera. Each amino acid was switched from Gpa-12 sequence back to Gα13 using PCR-based mutagenesis, to further characterize the amino acids causing loss of SRE-mediated growth and interactions with RH-RhoGEFs (*Methods 2.1*).

4. Discussion

The Gα12/13 subfamily of heterotrimeric G proteins is the only subfamily capable of causing oncogenic transformation in cultured fibroblasts [4]. This oncogenic nature has provoked investigation of the structural regions of Gα12/13 involved in growth signaling. Both Gα12 and Gα13 stimulate the SRE pathway via RhoA-dependent mechanism, while research has also shown that these two alpha subunits have unique mechanisms for activating SRE-mediated signaling [1,3,4]. Since Gα12 and Gα13 diverged in sequence following evolutionary G12/13 gene duplication, it is likely that their distinct growth signaling mechanisms are a result of various interactions with downstream proteins. X-ray crystallography of Gα12 and Gα13 in complex with effector proteins has proven challenging for researchers, because this method requires a copious amount of functional, homogenized Gα12/13 [8]. The only crystal structure of a G12/13 protein complex is Gα13 co-crystallized with p115RhoGEF [9]. Hajicek et al. identified multiple residues critical for Gα13 interaction with p115RhoGEF [9]. Due to the lack of crystal structures, mutagenic approaches have proven to be viable methods for identifying regions of Gα12 and Gα13 that interact with specific effector proteins.

Previous laboratory data has shown that the conserved C-terminal region is necessary for SRE-mediated growth signaling in both Gα12 and Gα13. However, the functional role of effector protein binding in the region has not been investigated. This project used a mutagenic approach to better define the functional properties of the Post-switch region and understand the different signaling methods that evolved. The Gα12/13 Post-switch chimeras both failed to activate SRE-mediated growth, which led this project to protein binding assays. The Gα13 Post-switch mutant showed inhibited interactions with the RH-RhoGEF proteins, while the Gα12 Post-switch mutant exhibited no change in binding. These data, therefore, suggest differing methods of growth signaling that evolved in Gα12 and Gα13 since diverging from a singular, evolutionary homolog like Gpa-12.

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 G12/13 alpha subunits [4,8]. Potential therapies for Gα12 or Gα13 driven cancers need to be able to target subunit-specific effector interactions in order to disrupt cell growth and tumor invasion. Thus, further characterization of the Post-switch region in Gα12 and Gα13 may contribute to the development of inhibitory drugs that could be used disrupt the various Gα12 or Gα13 specific interactions that lead to oncogenic activity.

5. Conclusions

The specific goals of this study were to identify critical residues of Gα13 necessary for SRF-mediated signaling and downstream protein interactions with RH-RhoGEFs. The motivation for the research was that previous data abolishment of SRF signaling when the Post-switch region in Gα12 and Gα13 was substituted with sequence from Gpa-12. Point mutations were made for each of the eight amino acids; two chimeras from Gα12/Gpa-12 suppressed SRE signaling while the analogous point mutations in Gα13/Gpa-12 had no effect. This led to my project to characterize the combination of amino acids necessary for Gα13 growth signaling through SRF and RH-RhoGEF interactions. Future work will include protein interaction assays and the creation of other chimeras with different amino acid combinations. Further exploration of the Gα13 and p115RhoGEF complex is needed, because the Post-switch region amino acids were not included in Hajicek et al. [9]. This research is important since Gα12 or Gα13 can be selectively overexpressed by certain cancers. It is crucial that we continue to identify key structural and functional differences between these two proteins as potential drug targets.

6. Acknowledgments

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