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Invertebrate Substitutions of a Highly Conserved Region in Gα13 Reveal Determinants of SRF Signaling and RhoGEF Binding

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Abstract

Gα12 and Gα13 are alpha subunits of heterotrimeric guanine nucleotide-binding proteins, and drive signal transduction pathways responsible for cellular growth, cytoskeletal alterations, and oncogenic development. Both proteins bind and activate a specific group of Rho-directed guanine nucleotide exchange factors (RhoGEFs) in order to stimulate several cellular responses, but the structural features that are distinct between Gα12 and Gα13 in this mechanism are not known. Invertebrates possess a single homolog of mammalian Gα12/Gα13, which retains the ability to trigger cytoskeletal rearrangements but is incapable of signaling to SRF (serum response factor), a transcriptional activator that plays a role in Gα12- and Gα13-mediated tumor progression. Previous research in our lab revealed a highly-conserved 36-amino acid region that contained only 10 (for Gα12) and 9 (for Gα13) divergent residues when compared to the Caenorhabditis elegans homolog. Although invertebrate substitution of this region eliminated SRF signaling for both Gα12 and Gα13, disruption of RhoGEF binding was observed only for the Gα13 mutant. To understand the Gα13-specific structural determinants of this interaction, we bisected its 36-residue region and found that the N-terminal invertebrate substitutions were crucial to abolishing RhoGEF binding and SRF signaling. However, single-point mutations in this region were inconsequential, leading to our current strategy of constructing multi-point invertebrate substitutions to subdivide the Gα13 N-terminal region and identify its critical residues for SRF signaling

and RhoGEF interaction. We have identified two mutants of Gα13, each containing three invertebrate substitutions, that show a dramatic loss of SRF signaling. Currently, co-precipitation assays using immobilized RhoGEF domains are being conducted to observe the impact of these mutations on binding to RhoGEFs and other known Gα13 target proteins.

Introduction

In order for a cell to properly function, the ability to detect the presence of extracellular signals and respond accordingly through a vast array of protein-mediated pathways is critical. To do so, the cell uses a class of receptors called G protein-coupled receptors (GPCRs), which use heterotrimeric G proteins to transduce information from ligand binding to downstream effectors to promote a specific cellular response. The G protein heterotrimer consists of a guanosine diphosphate (GDP) bound α subunit, which associates with the subunits β and ɣ at rest. The binding of a ligand such as a neurotransmitter, hormone, or odorant facilitates a conformational change in the GPCR, catalyzing the exchange of GDP for guanosine triphosphate (GTP) on the α subunit. The activated α subunit undergoes a conformational change, which allows it to dissociate from the βɣ dimer and interact with a vast array of downstream effectors until its intrinsic GTPase activity hydrolyzes GTP to GDP, inactivating it (Oldham and Hamm 2008). Four subfamilies of G protein α subunits exist: Gs, Gq, Gi, and G12/13. One of these families, G12/13 possesses the two proteins Gα12 and Gα13, which are known to drive several factors involved in cellular growth, migration, and cytoskeletal alterations (Suzuki et al. 2009). Gα12 and Gα13 are of clinical interest as they are the only G proteins known to drive oncogenic development and tumor metastasis in both a mutationally active form and in an overexpressed, wild-type state (Fromm et al. 1997, Rasheed et al. 2022).

One of the primary pathways in which Gα12 and Gα13 regulate cellular growth is through mediation of signaling to the transcription factor serum response factor (SRF), which when activated, initiates the transcription of genes possessing the serum response element (SRE) promoter region (Hill et al. 1995). SRE-mediated genes include many implicated in early cellular growth such as *c-Fos*, which is a known proto-oncogene (Yu et al. 2015). One of the primary SRF activation pathways is a Rho-mediated pathway, where activation of RhoA ultimately results in the nuclear localization of myocardin-related transcription factor A (MRTF-A), which serves as a coactivator of SRF (Figure 1). RhoA activation is stimulated by the exchange of bound GDP for GTP, which is carried out by a class of proteins called Rho guanine nucleotide exchange factors (RhoGEFs), which also happen to be common downstream effectors of Gα12 and Gα13 (Figure 1). Gα12 and Gα13, being 67% similar in sequence identity, are both known to bind to and stimulate the activation of RGS-homology(RH)-RhoGEFs, including p115-RhoGEF, PDZ-RhoGEF, and leukemia-associated RhoGEF (LARG) (Siehler et al. 2009). Much previous work has been done to elucidate the structural differences and mechanisms by which Gα12 and Gα13 interact with RhoGEFs, including chimeric substitutions of the sequence of a known invertebrate G12/13 homolog found in *Caenorhabditis elegans* (Gpa-12), which has 52% sequence identity

to Gα12 and Gα13 (Yau et al. 2003, Stecky et al. 2020). This invertebrate homolog appears to retain the ability to trigger cytoskeletal rearrangements within the cell, and is known to bind to similar downstream effectors such as CeRhoGEF. However, it is not involved in cellular growth pathways and is incapable of binding to mammalian RH-RhoGEFs, and thus cannot stimulate the mammalian SRF pathway. Sequence substitutions of Gpa-12 into mammalian Gα12 and Gα13 have been utilized to elucidate their key residues involved in SRF signaling and RhoGEF binding by making regional and point substitutions within both proteins, and observing if SRF signaling and/or RhoGEF interaction is disrupted by those mutations (Stecky et al. 2020).

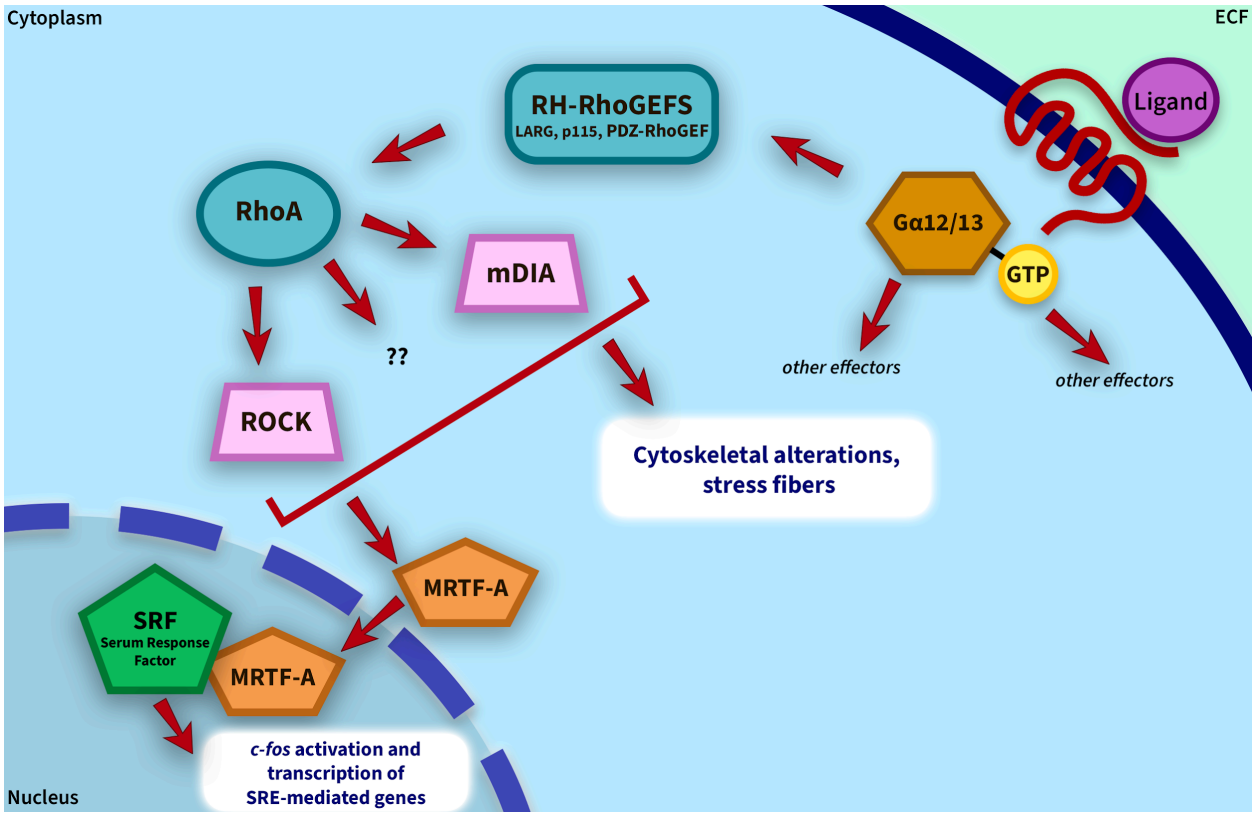


Figure 1. Serum response factor activation through a Gα12/13-mediated pathway. After guanine nucleotide exchange facilitated by the stimulation of the GPCR (red) by an extracellular ligand (purple), Gα12 and Gα13 both accept GTP (yellow), which results in their dissociation from the βɣ dimer. Gα12 and Gα13 interact and activate RH-RhoGEFs, which result in the activation of RhoA. RhoA activation promotes the activation of several downstream effectors, which subsequently results in MRTF-A (orange) relocating within the nucleus, where it associates with SRF (green). The formation of the MRTF-A/SRF complex results in the expression of SRE-mediated genes, including many implicated in cell growth.

A previous study focusing on aligning the sequences of Gpa-12, Gα12, and Gα13, identified the presence of a divergent region in the C-terminus of the proteins,

which is required for SRF signaling in Gα12, but not Gα13 (Stecky et al. 2020). While previous literature focused on determining the role of this divergent region in cellular signaling and RhoGEF interactions, they also identified a highly conserved region neighboring it, possessing only eight divergent residues in its 36-amino-acid-long sequence. This region was titled the “Post-Switch” region due to its position between the Switch III and divergent regions of Gα12/13. To determine the role of this Post-Switch region in RhoGEF binding and SRF activation, chimeras of Gα12/13 were constructed to substitute the region with its Gpa-12 equivalent via PCR-based mutagenesis. Subsequent SRE luminometry and protein co-precipitation assays demonstrated that the Post-Switch region plays a critical role in the ability of Gα12/13 to signal to SRF and bind to RH-RhoGEFs, with only Gα13 losing the ability to bind to RH-RhoGEFs. Bisection of this region to identify residues critical for this alternative mechanism of RhoGEF interaction was performed by generating substitution chimeras where the N-terminal portion of the Post-Switch region (possessing only five divergent residues) or the C-terminal portion (possessing four divergent residues) were replaced with the Gpa-12 homolog sequence (Stecky et al. 2020). Luminometry and co-precipitation assay results indicated that only the N-terminal portion of the Post-Switch region was essential for RhoGEF interaction and SRF signaling, as C-terminal substitutions did not impair signaling or RhoGEF interactions. To further investigate and elucidate which residues were critical for the signaling and effector interaction ability of the Post-Switch region, single-point invertebrate substitutions of each of the five divergent residues within the N-terminal portion were created and examined for their ability to signal to SRF. Unexpectedly, none of the single-point mutations significantly reduced the ability of Gα13 to signal to SRF, suggesting that SRF signaling in the Post-Switch region is dependent on a permutation of multiple residues.

The goal of this study was to determine further which residues were responsible for Gα13’s ability to stimulate SRF activation by creating multi-point invertebrate substitutions in the N-terminal portion of the Post-Switch region. Furthermore, we have sought to examine the impact of these mutations on binding to RH-RhoGEFs to elucidate the identity and the role of all RhoGEF-implicated residues within this portion of the Post-Switch region.

Materials and Methods

PCR-Derived Mutagenesis

Gα13 substitution mutants were engineered via polymerase chain reaction (PCR) mutagenesis. Engineered oligonucleotides were designed with the desired mutations and were added to a plasmid of myc-tagged, constitutively-active Gα13 (also known as Gα13QL-myc, possessing a Gln to Leu mutation) as a template (Eurofins Genomics, KY). The initial round of PCR created fragments that overlapped 18-20 base pairs and were isolated via gel electrophoresis. After purification via Wizard SV columns (Promega, Madison, WI), the purified fragments were utilized as templates for the second round of PCR. The length of each construct was verified via gel electrophoresis

and purified using the Wizard SV columns. All constructs alongside Gα13QL were digested using restriction enzymes NheI-HF and KpnI (New England Biolabs, Ipswich, MA) and cloned into the mammalian expression vector pcDNA3.1(+). The final products were confirmed by Sanger sequencing (Genewiz, NJ) to ensure that the amplified DNA construct possesses Gα13 with its desired modifications.

Serum Response Element Dual Firefly Luciferase Assays

Human embryonic kidney (HEK293) cells were grown in Dulbecco's Modified Eagle's Media (DMEM) with 10% fetal bovine serum and were passaged once they reached confluence. The cells were suspended using a 0.25% trypsin solution and were dispensed evenly into 12-well plates, and were incubated at 37 °C until the cells appeared to be at ~80-90% confluence under phase contrast microscopy. Each well of cells was transfected with 200 ng SRE-luciferase plasmid, 20 ng standard Renilla pRL-TK plasmid, and 200 ng of construct-encoding plasmid, Gα13QL, or pcDNA3.1(+). 2 µL of 2 mg/mL polyethyleneimine (Cold Spring Harbor, NY) was added to 70 µL of serum-free DMEM. Lysis of cells and SRE luminometry assays were performed approximately 48 hours post-transfection by washing cell samples in 1 mL of 1x PBS and treating them with 250 µL of 1x passive lysis buffer (Promega) while shaking at 120 rpm for 20 minutes. Lysates were pelleted by centrifuging them at 16,000 x *g* for 1 minute, and supernatants were analyzed via dual-luciferase assay and GloMax 20/20 luminometer (Promega). Luminescence of each sample was measured twice, once after catalyzing the reaction of SRE-mediated firefly luciferase, and again after both quenching the firefly luciferase reaction and stimulating Renilla luciferase activity. Firefly luciferase activity was quantified by measuring the intensity of sample luminescence, which was normalized for Renilla luciferase activity to account for possible variations in transfection efficiency, and the ratio of firefly luciferase luminescence to Renilla luciferase luminescence was interpreted to determine the presence and strength of SRF signaling. Samples of each supernatant before the dual-luciferase assay were saved for analysis via SDS-PAGE and immunoblotting.

GST Co-Precipitation Assays for GST-RhoGEF Interactions

BL21(Gold)-DE3 cells were transformed with a cDNA-containing plasmid encoding for an array of RH-RhoGEFs conjugated to a glutathione-S-transferase (GST) tag (Agilent, Santa Clara, CA). Cells possessing plasmids encoding for either GST-p115RhoGEF, GST-LARG, GST-PDZRhoGEF, or GST alone were grown in Luria-Bertani (LB) broth with 75 ug/mL ampicillin at 37 °C, 220 rpm for approximately 14 hours. Afterwards, for each GST-RhoGEF fusion construct, 6 mL of their respective cultures were added to 500 mL of LB with 75 µg/mL ampicillin and were incubated at 37 °C, 220 rpm until an A_{600} within 0.5-0.8 was measured.
Isopropyl-β-D-thiogalactopyranoside (Fisher Scientific, Pittsburgh, PA) was added to each 500 mL culture at a concentration of 0.5 mM and left to incubate for 3.5 hours to stimulate GST-RhoGEF expression. Cultures were then spun at 6000 x *g* for 15 minutes to pellet the cells, which were then resuspended in 2.5 mL of chilled 2.3 M sucrose, 50 mM Tris pH 7.7, 1 mM EDTA alongside protease inhibitors phenylmethylsulfonyl fluoride

and *Na*-tosyl-L-lysine chloromethyl ketone. Samples were diluted using 10 mL of 50 mM Tris pH 7.7, 10 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol, and cells were subsequently lysed using 4.5 mg of lysozyme (MP Biomedicals, Santa Ana, CA) on ice for 1 hour. Sodium deoxycholate (0.1% w/v), 20 mM MgCl2, and 10 µg/mL of DNase I were added to lysates, which were then incubated on ice for 10 minutes and centrifuged at 17,000 x *g* at 4°C for 40 minutes. Pre-washed glutathione Sepharose 4B beads (GE Healthcare Biosciences, Pittsburgh, PA) were added to the supernatants for 45 minutes at 4°C to facilitate precipitation of the GST-RhoGEF fusions after centrifugation at 1300 x *g* for 3 minutes. The resulting pellets were then washed twice in 50 mM Tris pH 7.7, 1 mM EDTA, 1 mM dithiothreitol, and 150 mM NaCl, and aliquots were snap-frozen in liquid N_2 for storage at -80°C.

Lysates for each Gα13 mutant alongside Gα13QL were produced in preparation for the co-precipitation assays. HEK293 cells were grown in 10cm plates and were transfected using 5.0 µg of the appropriate plasmid alongside 0.5 mL of DMEM. Plates were grown to approximately 80% confluency. After incubation at 37 °C for approximately 38-42 hours, cells were washed twice in PBS, vigorously scraped from the dish, and then pelleted by centrifugation at 500 x *g* for 5 minutes. Pellets were resuspended using 50 mM HEPES pH 7.5, 1mM EDTA, 3 mM dithiothreitol, 10 mM MgSO4, 2.5% polyoxyethylene-10-lauryl ether (LPX) enhanced with 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (0.84 mM), leupeptin (1.1 uM), pepstatin (0.73 uM), *Na*-tosyl-L-lysine chloromethyl ketone (58 uM), tosyl-L-phenylalanylchloromethane (61 uM) and phenylmethylsulfonyl fluoride (267 uM). Lysates were inverted at 4 °C for 30 minutes and then centrifuged at 90,000 x *g* for 1 hour. Supernatants were aliquoted and snap-frozen in liquid N_2 for storage at -80 °C.

Co-precipitation assays were conducted by diluting lysates for each construct, Gα13QL, and a “blank” lysate possessing no Gα13 expression twenty-fold by using HEDM buffer (comprised of 50 mM HEPES pH 7.5, 1 mM EDTA, 3mM dithiothreitol, and 10 mM MgSO4) in preparation for the later addition of LPX used in extraction. 30 uL of diluted lysates were saved from each lysate aliquot as “load” samples to serve as a positive control for cellular protein expression levels for each of the constructs. GST-RhoGEF fusions were combined alongside the rest of the lysates and were continuously inverted at 4°C for 2 hours. Samples were centrifuged at 1300 x *g* for 3 minutes and washed twice in HEDM buffer supplemented with 0.05% LPX. Proteins were pelleted at 1300 x *g* and saved for analysis via SDS-PAGE and immunoblotting. 3 uL of each interaction sample was saved and analyzed via SDS-PAGE and Coomassie Brilliant Blue (CBB) staining to determine the presence of GST-RhoGEF fusions in each sample.

SDS-PAGE and Immunoblotting

After SRE luciferase and GST co-precipitation assays, samples were analyzed for protein expression by conducting sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting. Cell lysates were denatured in 4x Laemmli buffer containing 0.1M dithiothreitol at 72°C for approximately ten minutes. The prepared samples were then run on a 12% polyacrylamide gel at 135 volts. Samples were

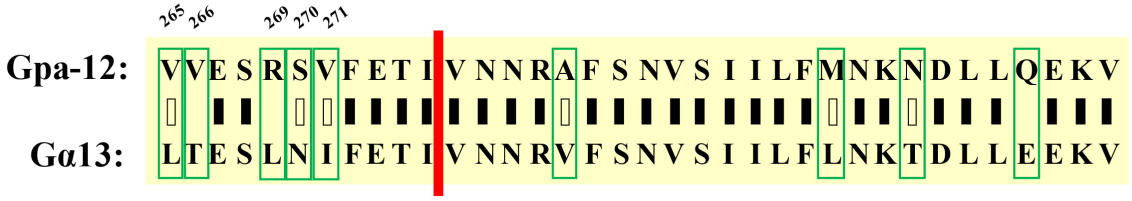
transferred from the polyacrylamide gel to a nitrocellulose membrane for immunoblotting at 4°C, 35 volts overnight. Protein blocking was achieved using a 5% w/v powdered milk-TBST solution. Blots were probed using anti-Gα13 (Millipore Sigma) polyclonal primary antibodies, mixed at a 1:2000 dilution factor in milk-TBST, followed by secondary anti-mouse antibodies conjugated to alkaline phosphatase (Promega) mixed at a 1:7500 dilution factor in milk-TBST. Western blots were developed using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in an alkaline environment in which the antibody-conjugated alkaline phosphatase catalyzes the production of a blue-violet formazan derivative. Protein expression was imaged using a Kodak Gel Logic 100 system alongside CareStream molecular imaging analysis software (Rochester, NY). Gα13 presence was determined by observing the absence or presence of bands near 45 kDa relative to a known protein standard.

Results and Discussion

N-terminal Triple-Point Mutations Nearly Abolish SRF Signaling

To further determine which N-terminal residues of the Post-Switch region are required for SRF signaling and RhoGEF binding, the generation of mutants possessing invertebrate substitutions of the divergent residues within this region is necessary, as the decoupling of mutated Gα13 from the SRF pathway would suggest that critical residues important for interacting with this pathway had been changed or replaced. Multiple residues contribute to the ability of the Post-Switch region to stimulate this pathway, as single-point mutations of each of the divergent residues within this region still strongly retained the ability to signal to SRF. Therefore, we sought to generate double and triple-point mutations that substituted two or three of the divergent residues in Gα13 with their Gpa-12 variants based on sequence alignment data (Figure 2A). Four chimeras based on the sequence of Gα13QL were generated: a mutant possessing Leu^{265} to Val^{265} and Thr^{266} to Val^{266} substitutions (L265V;T266V), a mutant possessing Asn^{270} to Ser^{270} and Ile^{271} to Val^{271} substitutions (N270S;I271V), and triple-point mutations of both of the aforementioned constructs to include the change of Leu^{269} to Arg^{269} (L265V;T266V;L269R and L269R;N270S;I271V respectively) (Figure 2B).

A)



B)

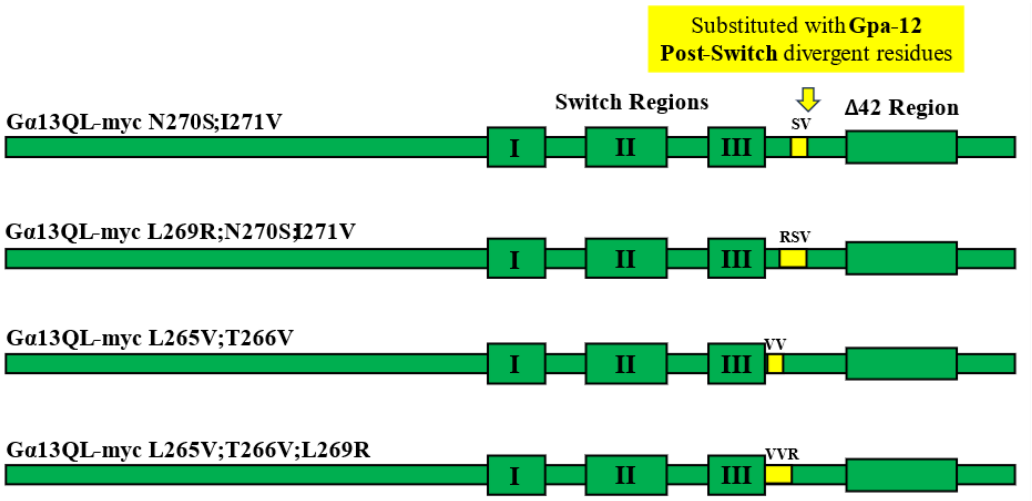
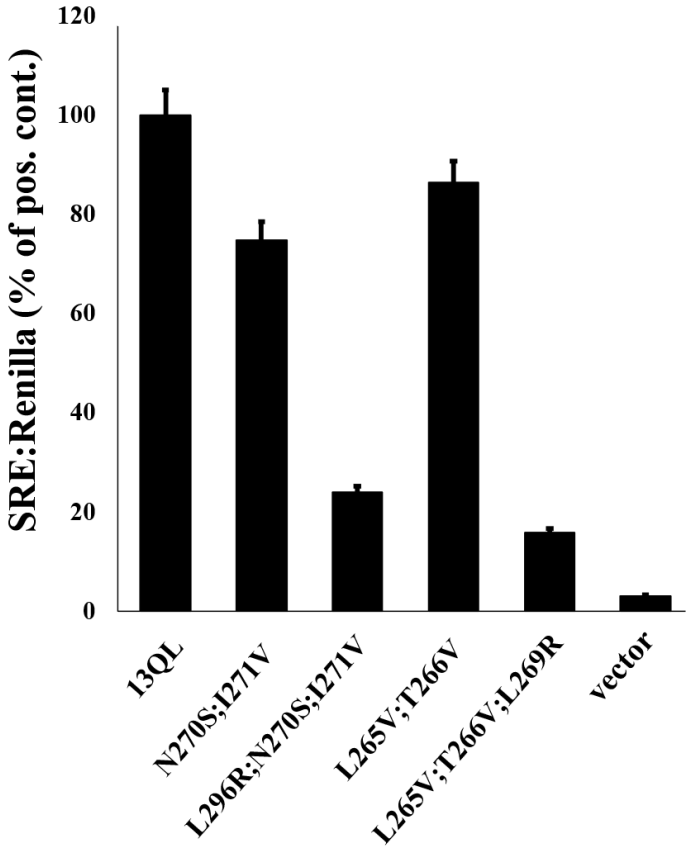


Figure 2. A) Aligned sequences of Gpa-12 and Gα13 within the Post-Switch region. Green boxes demarcate divergent residues between the two proteins. The red bar indicates the divide between the N-terminal portion of interest and the C-terminal portion of the Post-Switch region. Black boxes indicate exact sequence conservation between the two proteins, whereas empty boxes represent chemical property conservation between the two sequences. B) An illustrated representation of double and triple-point Gpa12 substitutions in the Post-Switch region of Gα13.

SRE Dual-Luciferase assays were conducted on each of the four mutants to determine their ability to signal to SRF and stimulate the expression of SRE-mediated genes. Double-point mutations present in constructs L265V;T266V and N270S;I271V appeared to strongly retain the ability of Gα13 to signal to SRF, with ~86% signal intensity for L265V;T266V and ~75% signal intensity for N270S;I271V compared to constitutively-active Gα13 (Figure 3A). Conversely, triple-point mutations present in constructs L265V;T266V;L269R and L269R;N270S;I271V almost completely abolished the ability of Gα13 to signal to SRF, with ~16% signal intensity for L265V;T266V;L269R and ~24% signal intensity for L269R;N270S;I271V compared to constitutively active Gα13 (Figure 3A). Western blot analysis confirmed the presence of all construct proteins in their respective passive lysate samples, but bands of slightly lower intensity were noted for both triple-point mutants (Figure 3B).

A)



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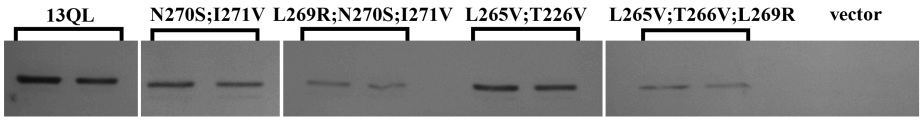

Figure 3. A) SRE signaling strength of double and triple-point mutations relative to Gα13, as measured by luciferase assay. Values are displayed as the average percentage of the mean positive control value (set at 100% in each experiment) ± standard error of the mean. B) Western blot analysis of Gα13 mutants compared to Gα13QL and pcDNA3.1(+) (vector). Immunoblotting data verify the presence of protein in multiple replicates of the luciferase assay.

GST Co-Precipitation Assays Yield Inconclusive Results

Co-precipitation assays were conducted to determine if double and triple-point substitution mutants could bind to RH-RhoGEFs. However, immunoblotting data for all mutants appeared to be inconclusive. For all mutant lysates, all bands, including “load” samples, could not be detected via immunoblotting. Bands were visible for Gα13 at 45 kDa for p115RhoGEF, PDZRhoGEF, and LARG interaction samples, as well as the “load” positive control, but were noticeably faint (Figure 4A). Coomassie Brilliant Blue staining confirmed the presence of all GST-RhoGEFs in their respective interaction samples (Figure 4B).

A)

B)

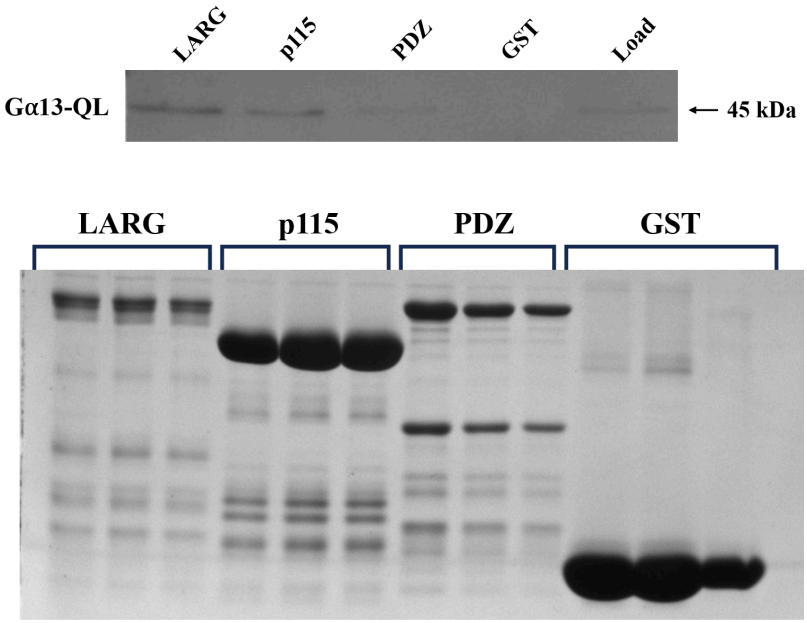

Figure 4. A) Western blot analysis of Gα13QL across all GST-RhoGEF interaction samples. Bands are present at 45 kDa for LARG, p115RhoGEF, and PDZRhoGEF in addition to the “load” positive control, suggesting that Gα13QL was present. B) Coomassie Blue analysis of GST-RhoGEF fusions indicates presence of GST-RhoGEF fusions in all interaction samples.

Discussion and Future Work

As triple-point substitutions in the N-terminal portion of the Gα13 Post-Switch region dramatically reduce its ability to signal to SRF and facilitate the expression of SRE-mediated genes, our findings suggest that the presence of at least three of the five divergent residues is required to allow SRF activation by Gα13. Interestingly, each one of these triple-point mutations possessed a Leu^{269} to Arg^{26}, a mutation that by itself does not significantly disrupt signaling to SRF, but appears to diminish signaling the most out

of all single-substitution mutants (Stecky et al. 2020). Further investigation to determine if Leu^{269} plays any significant role compared to the other divergent residues is needed to fully confirm if SRF signalling and RhoGEF binding are dependent solely on the number of divergent residues present in the N-terminus of the Post-Switch region. More double-point mutants possessing the Leu^{269} to Arg^{26} mutation and more triple-point mutants lacking it are planned for development and further analysis.

Unfortunately, co-precipitation assays conducted to determine if double and triple-point substitution mutants could bind to RH-RhoGEFs yielded largely inconclusive data, as the inability to detect mutant Gα13 hindered our ability to confirm or deny protein interactions. Coomassie blue staining results indicate that GST-RhoGEF fusions were present in all interaction samples, suggesting that the “pulldown” procedure for the co-precipitation assays was performed correctly. Instead, the issue appears to lie in the reagents used in creating the lysates, growing and transfecting the HEK293 cells, and/or immunoblotting. Furthermore, several freezer crashes and equipment failures impeded our ability to re-attempt these co-precipitation assays, as all construct lysates and GST-RhoGEF fusions were compromised. Several steps have already been taken in an attempt to rectify these issues. All reagents involved in HEK293 cell growth, transfection, and lysis were remade. Notably, fetal bovine serum from a different vendor (Corning, Glendale, AZ) improved HEK293 growth rates and confluence. Additionally, increasing the concentration of LPX to 2.5% (w/v) appears to yield a higher amount of soluble Gα13 in the lysate when compared to the original concentration (Fowler, 2024). A new anti-GNA13 antibody (Millipore Sigma) was purchased to replace an older stock used to develop the blots in this study. All Gα13 constructs contain a myc epitope tag, an anti-myc primary antibody may be used if blotting with the new anti-GNA13 stock is unsuccessful. Furthermore, all GST-RhoGEF fusions were recreated using their respective, pre-existing BL21(Gold)-DE3 stocks. Co-precipitation assays with remade reagents and GST-RhoGEF fusions are currently being conducted to investigate whether triple-point substitution mutations disrupt the ability of Gα13 to bind to RH-RhoGEFs.

Future studies have the potential to investigate the impact of triple-point mutations further by generating triple-point mutations not present in this study, including triple-point mutations that do not possess the Leu^{269} to Arg^{269} mutation found in both triple-point mutants used in this study. In Western blot analysis, lower intensity bands were noted for both triple-point mutants. While it is possible that protein expression levels could have minimally impacted the results of the SRE luciferase assays, we believe that the slightly lower intensity of these bands does not fully account for the approximate 80% loss of signaling to SRF that both mutants exhibited. An alternative explanation could be that these triple-point mutations disrupted the structure in which the stability of the protein itself was compromised, impacting both detection via a polyclonal antibody and its ability to signal to downstream effectors in the SRF pathway. To further investigate this, an alternative antibody, such as the anti-myc antibody, could be used in immunoblotting to detect the presence of mutated Gα13. Additionally, it is possible that these mutations could make it more difficult for Gα13 to be stripped away from the membrane, thus decreasing the amount of soluble Gα13 available. To rule this out, cellular fractionation assays could be conducted to investigate further the impact of these mutations on the cellular localization of Gα13. Furthermore, future studies

designed to identify structural differences between Gα12 and Gα13 in the Post-Switch region, in conjunction with the results of this study, may be beneficial in contributing to the design and development of more accurate drug targeting of these proteins, which could have implications for cancer therapeutics and treatments.

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