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Mutation of Class-Distinctive Residues in Gα13 to Identify Determinants of RH-RhoGEF Binding Selectivity

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Abstract

Gα13 belongs to the Gα12/13 subfamily of heterotrimeric guanine nucleotide-binding proteins, which play a signaling role in cell growth and tumorigenic pathways, cytoskeletal rearrangements, and metastatic invasion. A structural bioinformatics analysis[1] identified a set of "class-distinctive" residues in Gα13 which correspond to a different residue conserved in the other G protein subfamilies: Gs, Gi, and Gq. Using this information, we created a panel of Gα13 point mutants that replace each class-distinctive residue with its putative ancestral form. In order to distinguish mutant constructs from native Gα13 in cultured human embryonic kidney cells, a myc epitope tag was introduced to all Gα13 mutants, positioned within the αB–αC loop of the helical domain. Installation of this epitope tag was non-disruptive to Serum Response Factor signaling by Gα13 and allowed for differentiation of the recombinant and native forms in protein-protein interaction experiments. While characterization of these Gα13 mutants is ongoing, there is a class-distinctive Phe at position 234 for which ancestral substitution appears to cause selective uncoupling of protein binding within the Gα13-responsive, RGS-homology (RH) RhoGEF. These findings shed light on the mechanism of Gα13 interaction with the individual RH-RhoGEFs - p115RhoGEF, PDZ-RhoGEF, and leukemia-associated RhoGEF - and further suggest that class-distinctive Gα13 mutants may reveal binding determinants for additional effector proteins.

1. Introduction

The Gα12/13 family of heterotrimeric G proteins are pivotal in facilitating cellular events including growth, cytoskeletal arrangements, cell migration, and adhesion[3]. Being a nexus of cancer-related pathways, it is no surprise that the deregulation of these proteins are implicated in a range of cancers including prostate, breast, and esophageal[5]. Cancers associated with a faulty Gα12/13 axis tend to favor one subunit over the other, illustrating the unique character and fidelity these α subunits have. For instance, a Gα13-chemokine receptor axis plays a key role in the migration of breast cancer cells[6].

Like other heterotrimeric G proteins, these α subunits form a complex with β and γ subunits at the intracellular surface of a G-coupled protein receptor (GPCR). Once these transmembrane receptors are stimulated by the binding of an extracellular ligand, the coupled cytoplasmic alpha subunit exchanges GDP for GTP, becoming activated. The activated subunit then dissociates from the βγ dimer and localizes into the cytoplasm where it is free to associate with downstream targets.

A particular set of downstream targets for Gα13, the rgRGS-Rho guanine exchange factors (RH-RhoGEFs) are of particular interest as they have been shown to have a unique role in signaling Rho-associated events, including cytoskeletal rearrangements and cell cycle regulation, through their interaction with the Gα12/13 family[4]. These RH-RhoGEF proteins are capable of acting as both a factor in guanine-nucleotide exchange, and a GTPase-activating (GAP) protein[7]; essentially, being able to "turn on" Rho-associated events while also capable of accelerating the "turning off" of its upstream α subunit. This GAP functionality is imparted due to the presence of a conserved regulatory g protein signaling (RGS) domain[7]. This functionality is of pivotal importance in understanding

heterotrimeric G proteins in the context of oncogenesis, as the rate of GTP hydrolysis of activated α subunits dictates the duration and amplitude of GPCR signaling[7].

A bioinformatics paper published in 2010 by Temple et. al aligned the entire heterotrimeric G protein family using their knowledge of conserved domains important to the characteristic functionality of g proteins. Results of this alignment generated a panel of amino acids that are thought to be evolutionarily characteristic of the heterotrimeric G protein family to which it belongs.

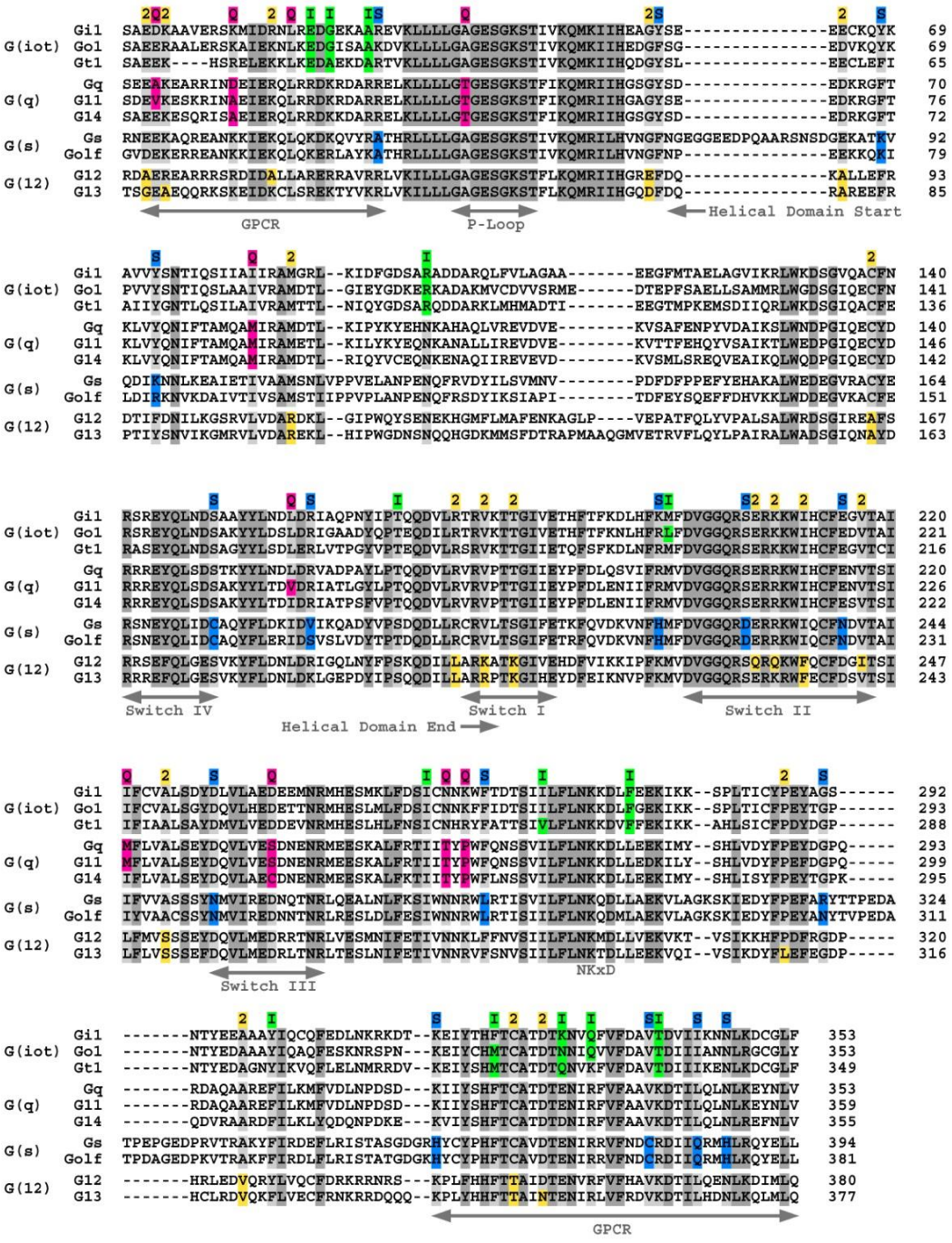

Figure 1. Class-distinctive mutations in the heterotrimeric G protein family.

Figure 1. Graphic of the entire heterotrimeric G protein family and their respective class-distinctive mutations courtesy of Meigs' Lab collaboration with Brenda Temple[1]. Highlighted yellow amino acids are designated as a class-distinctive mutation in the Gα12/13 family. The three Switch regions are loops of alpha-helices and are conformationally sensitive to guanine nucleotide binding. The P-loop is crucial for binding GDP/GTP and is a highly conserved region.

In order to further study the significance of designated amino acids in Gα13, class-distinctive mutants in which the designated class-distinctive amino acid is replaced with its putative, ancestral version, were created. A myc epitope tag was then inserted into these class-distinctive mutants to provide them extra molecular weight, thus allowing for the differentiation of native vs wild-type Gα13 cell lysates. This myc-tag was inserted between the αB–αC loop of the helical domain for 13 of a possible 15 class-distinctive mutations for Gα13. In order to assure there were no functional consequences due to the insertion of the myc tag, the myc-tagged mutants were assessed using a gene reporter assay measuring Serum Response Factor (SRF) activity. The activity of this SRF protein is associated with the upstream activity of the Gα12/13 protein family, and as such, makes for a useful tool in assessing their functionality.

The F234I myc-tagged mutant was further analyzed for binding perturbations with the three RH-RhoGEFs: LARG, PDZ-RhoGEF, and p115-RhoGEF. Their unique and conserved functional domain (rgRGS) makes these RH-RhoGEFs interesting targets to assess practical consequences of these evolutionarily characteristic class-distinctive amino acids. Previous work on Gα12 mutational analysis in the N-terminal region has shown selective RH-RhoGEF uncoupling[8]. It is hypothesized that these class-distinctive Gα13 mutants may elucidate further selective RH-RhoGEF determinants.

In this investigation, we show that a single Phe to Ile mutation in Gα13 abolishes its ability to bind p115, while retaining its characteristic interaction with LARG and PDZ-RhoGEF. This is interesting as previous results have suggested that the Gα13 and p115 interaction is unique even within the RH-RhoGEF family, with p115 and Gα13 interacting at a known effector binding pocket, instead of the usual SwitchI region[7]. Further assessment of binding perturbations between these class-distinctive Gα13 mutants and their RH-RhoGEF interactors is ongoing.

2. Materials and Methods

2.1 DNA Constructs

The A161C Gα13 point mutant was created utilizing PCR-based mutagenesis. A pair of amplimers were created based on WT Gα13 sequence and contained a 19-20 bp overlap between them. The template for Gα13 that was used contains a myc-tag and is artificially activated through its QL mutation.

All other point mutations in Ga13, previous engineered by Alicia Tagliatela in the Meigs lab, were further engineered to install a myc epitope tag that was in the αB–αC loop of the helical domain. This was done by digesting each Gα13 class-distinctive point mutant while, at the same time, digesting a Gα13-QL-myc counterpart to act as a myc-tag vector. Different endonucleases needed to be used for different mutations depending on where the mutation was in the gene.

Once digested, the class-distinctive Gα13 mutant inserts were separated by agarose gel electrophoresis and subsequently purified. The class-distinctive fragments were then combined with their myc-donor vector and ligated.

1 uL of these ligations were added to ~10 uL of competent JM109 *E. coli* cells and subsequently heatshocked in a heat bath at 42°C bath for 45 sec before being incubated on ice ~2min. 300 uL of SOC medium was then added to the cells and were allowed to grow at 37°C for 1h. These cultures were then pelleted by spinning at 9000g for 3 min before having all but ~100 uL of supernatant removed. The pellet was then resuspended in the remaining ~100 uL of supernatant and plated onto LB-Ampicillin plates. These plates were allowed to grow for ~14 h at 37°C.

Colonies that were produced were screened for the presence of a viable ligation by using a PCR diagnostic: one primer sits inside the class-distinctive insert while pointing to another primer that is located within the vector. If the insertion was ligated in reverse, no PCR fragment would be produced.

Correctly ligated mutants were then chosen for amplification and were allowed to grow in a 2 mL liquid culture of LB-Broth for ~14 h before being pelleted at 9000 x g for 5 min and having their supernatant removed. In order to further purify the plasmid, the resulting pellet was then combined with 250 uL of Qiagen P1 buffer and triturated inside a microfuge tube. After, 250 uL of Qiagen P2 (lysis buffer) was added to the tube, which was then inverted several times to mix and allowed to sit for 5 min. 350 uL of N3 (neutralizing buffer) was then added to the tube and inverted 20x. The tubes were then centrifuged at ~16,000 x g for 10 min.

Once pelleted, the supernatant (~850 uL) was transferred a Qiagen DNA affinity column and spun at 16,000 x g for 60s. The columns were then washed with 0.5 mL of Qiagen PB Buffer, and 0.75 mL of Qiagen PE (wash buffer), spinning at 16,000 x g after each. 50 uL of Qiagen EB (Elution Buffer) was added to each tube, let stand 3 min, and then spun for 60s at 16,000 x g to elute plasmid DNA. All mutants were verified by sequencing (Genewiz, South Plainfield, NJ). All constructs were spliced into PcDNA3.1(+).

2.2 Preparation of Detergent-Soluble Proteins

Human embryonic kidney cells (HEK293) were grown in Dulbecco's modified Eagle's medium with a 10% solution of fetal bovine serum added to help supplement the cells. Polyethylenimine (PEI) was used to transfect 5µg of mutated DNA construct into cells when ~50% confluent. The cells were grown to 90% confluency before being harvested. Cells were washed in 1X PBS and scraped off of plate and into a solution of 1X PBS. This cell suspension was centrifuged 500 x g for three minutes. The resulting pellet was resuspended 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 1X Metalloprotease inhibitor. Lysates were consistently rotated for ~30 minutes before being spun 80,000 x g for 1h 30m. The resulting supernatants were snap frozen in liquid nitrogen before being stored at -80°C.

2.3 Protein Interaction Assays

50 uL of cell lysate extracts from transfected HEK293 cells were added to 800 uL of HEDM buffer [50 mM HEPES pH 7.5, 1 mM EDTA, 3 mM dithiothreitol, 10 mM $MgSO_4$] to dilute detergent concentration. 30 uL of this solution was saved prior to interaction experiment to be used as a control. Sepharose-bound GST fusion interactor proteins were diluted with HEDM buffer before being combined with HEK293 cell lystates. The resulting solution of combined interactors was allowed to mix for 90 min at 4°C. After, the interaction tubes were spun 1300 x g and washed twice with HEDM buffer containing 0.05% polyoxyethylene-10-lauryl ether to isolate the sepharose-bound complexes. These isolated samples were then subject to SDS-PAGE and immunoblotting using a primary Gα13 antibody and anti-rabbit secondary antibody. BCIP and NBT were used for developing blots.

2.4 Gene Reporter Assays

HEK293 cells were grown to ~80% confluency in 12-well plates before being 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 Gα13-QL-myc, or a class-distinctive Gα13-QL-myc 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. Lysates were analyzed 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.

3. Results

3.1 Myc-tag installation is nondisruptive to SRE signaling in Gα13-QL class-distinctive mutants

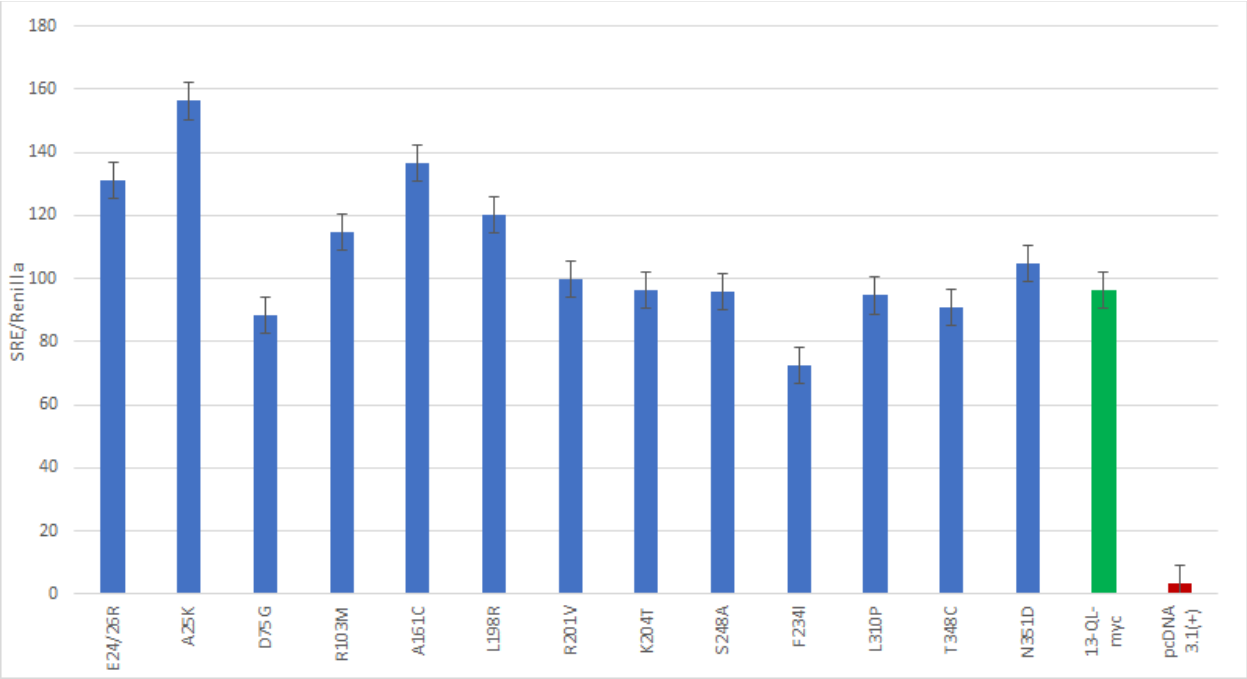

Figure 2. SRF activity of myc-tagged Gα13 class-distinctive mutations.

Figure 2. Gα13-QL-myc class distinctive mutations (blue) show no loss of SRF function. Gα13-QL-myc (green) was used as a positive control. pcDNA3.1(+) vector (red) was used as a negative control. All encoded proteins contain an activating Glutamine to Leucine (QL) mutation, which abolishes GTPase activity.

Wanting to assure that there were no functional consequences of myc-tag installation, the class-distinctive Gα13 mutants underwent a gene reporter assay to assess their ability to stimulate Serum Response Factor (SRF). This SRF is a transcription factor that is downstream of the Gα12/13 family that binds to SRE promotor regions and stimulates the transcription of various oncogenes. With the luciferase reporter plasmid's built-in SRF promotor, it is capable of assessing the relative activity of these class-distinctive Gα13 mutants, shown in Figure 2.

Previous results with myc-tagging Gα12 class-distinctive mutants have shown that that the tag is benign in altering functionality, which can also be seen in these Gα13 class-distinctive mutants (Fig. 2). It was also shown previously[3], that there was a loss of SRF function in particular Gα12 class-distinctive mutants, which does not seem to be replicated in these Gα13 mutants.

3.2 Gα13 F234I class-distinctive mutation shows selective uncoupling of p115-RhoGEF

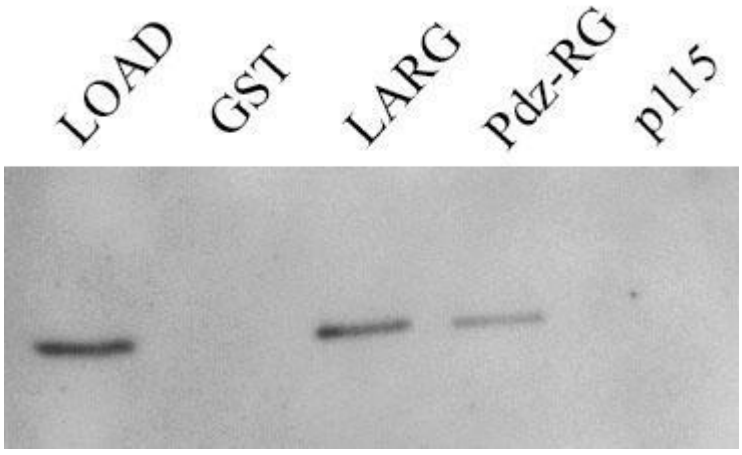

Figure 3. F234I-QL-myc Western Blot.

Figure 3. The F234I-QL-myc class-distinctive Gα13 mutant shows a selective uncoupling of p115-RhoGEF while retaining its characteristic binding to LARG, and PDZ-RhoGEF.

The resulting Western blot of the F234I myc-tagged mutant shows a definitive loss of coupling to p115-RhoGEF. The mutant retained its binding with the other two RH-RhoGEFs: LARG and PDZ-RhoGEF

4. Conclusion

The Gα12/13 family of heterotrimeric G proteins couple themselves to various GPCRs which, when activated, cause the Gα subunit to become activated and dissociate from its dimer. This activated subunit is then free to transduce its signal through downstream targets. The dysregulation of these proteins are associated with many types of cancer[6], and are unique in their ability to facilitate oncogenic transformation through mere overexpression of the wildtype[9]. A key determinant for Gα12/13's ability to produce these oncogenic effects is through their eventual activation of Rho.

One such association with the rgRGS-RhoGEFs is crucial in the Gα12/13 family being able to stimulate Rho activity. In order to study any possible effects of our class-distinctive Gα13 mutants, a myc tag was installed within the αB–αC loop of the helical domain. This panel of Gα13 myc-tagged mutants were then tested in a gene reporter assay to measure SRF activity against a control to assure there were no detriments to functionality after myc-tag installation.

After assaying the interaction between the Gα13 F234I class-distinctive mutant and the three rgRGS-RhoGEFs (LARG, PDZ-RG, and p115-RG), a loss of coupling was found with regards to the p115-RG. It appears the Phe to Ile mutation at amino acid 234 in Gα13 causes it to selectively uncouple from p115, while retaining its characteristic binding to LARG and PDZ-RhoGEF. This is interesting, as molecular crystal data has suggested that the Gα13 and p115 interaction was unique within the g12/13 family[10]. Further assessment of this class-distinctive mutant for possible perturbations in its rate of GTP hydrolysis in the presence of p115-RG may be one way to explain this result.

Previous work in Gα12 has shown that mutation of the N-terminal region can produce a selective loss of binding to LARG, while producing minimal effects on p115-RG binding[3]. This served as a precedent for the utilization of mutation to explore selective Gα13/rgRGS-RhoGEF uncoupling in attempting to discern the suggested non-redundant roles in the Rho signaling that they facilitate.

In another study, it was shown that Gα12 can signal Rho by binding to a region on AKAP-Lbc (another RhoGEF)[11]. This region is closely homologous to a region in p114 that was also found to interact with Gα12. Interestingly, this interaction was distinct from the typical RH-RhoGEF interaction, and was suggested to be interacting at another site on Gα12. It is interesting that this distinct interaction is displayed by both Gα12 and Gα13 and their interaction with p114 and p115, respectively.

Further exploration of the class-distinctive Gα13 mutants and the RH-RhoGEFs may elucidate more binding determinants for their interaction. Functional analysis of the F234I mutant may help to resolve our understanding of the exact roles these RH-RhoGEFs have in facilitating Gα12/13 effects.

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