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Analysis Of Class-Distinctive $G\alpha_{13}$ Mutants To Identify Regions Critical For Serum Response Element-Mediated Transcriptional Activation

Alicia C. Tagliatela[1], Tyler P. Hamilton[1], Alan M. Jones[2],
[1]Department of Biology, [2]Department of Biology and Pharmacology
University of North Carolina at Asheville
Asheville NC 28804

[2]University of North Carolina
Chapel Hill, NC 27599

Faculty Advisor: Dr. Thomas E. Meigs

Abstract

Guanine-nucleotide binding proteins (G proteins) are involved in transmitting signals from cell surface receptors to downstream pathways. The G12/13 class of G proteins includes $G\alpha_{12}$ and $G\alpha_{13}$, which diverged from a single ancestral G12/13 protein during evolution. These proteins drive proliferation and migration in certain cell types, and also regulate cytoskeleton rearrangements and other cellular events. In order to identify key signaling determinants that evolved in the G12/13 class, we engineered mutants in which class-distinctive residues in $G\alpha_{13}$ were substituted for residues conserved at the same structural position in the non-G12/13 classes of $G\alpha$ proteins. These mutants were transfected into human kidney cells, and serum response element (SRE) activation assays identified several substitutions in $G\alpha_{12}$ that hindered SRE activation. Surprisingly, the corresponding mutations in $G\alpha_{13}$ showed no significant reduction in this serum response. Furthermore, we engineered chimeras in which $G\alpha_{12}$ and $G\alpha_{13}$ incorporated regions from the fruit fly G12/13 homolog, Concertina, and found a C-terminal region for which substitution of Concertina sequence abolished $G\alpha_{12}$ signaling through SRE yet was fully tolerated in $G\alpha_{13}$. These findings suggest $G\alpha_{12}$ and $G\alpha_{13}$ utilize distinct structural mechanisms for engaging the pathway leading to SRE-mediated transcription.

1. Introduction

Heterotrimeric guanine nucleotide binding proteins (G proteins) are found in almost every tissue of the human body. They mediate several communication pathways within cells, regulating functions that include cell division, migration and metabolic processes when activated by a GDP-GTP exchange. When bound to GTP, the α subunit dissociates from the βγ dimer and acts as a signaling intermediate until one phosphate is removed by GTPase activity and it is returned to its inactive, bound form[1].

In 1991, Strathmann and Simon defined $G\alpha_{12}$ and $G\alpha_{13}$ as their own class of G protein, which share 67% gene identity with each other and less than 45% with other classes of G proteins[4]. There are many known effectors of both proteins in this class, and others that activate one but not the other. Rho guanine nucleotide exchange factors (RhoGEFs) are probably the most well studied of these activators, and have been described as activators of both $G\alpha_{12}$ and $G\alpha_{13}$. RhoGEFs have been identified as necessary intermediates for the activation of serum response element (SRE)-mediated transcriptional activation for $G\alpha_{12}$ and $G\alpha_{13}$[3], which is critical during embryonic development and skeletal muscle formation. The end of this pathway leads to an activation of serum response factor, which acts as an activator for a promoter region. A substitution of glutamine residue for a leucine residue will make both $G\alpha_{12}$ and $G\alpha_{13}$ constitutively activated ($G\alpha_{12}$QL and $G\alpha_{13}$QL), driving this pathway.

Comparison work done by Dr. Alan Jones has identified distinctive amino acids within the sequence of $G\alpha_{12}$ and $G\alpha_{13}$, and work has been done changing those class-distinctive amino acids to the ancestral form in $G\alpha_{12}$. A large region was distinguished as class-distinctive in both proteins near the C-terminus, and a chimera between $G\alpha_{12}$ and an ancestral G12 protein, Concertina, replaced this region with a sequence more traditionally found in G proteins. These mutants were tested for activation of serum response factor (SRE) against the wild type and activated form ($G\alpha_{12}$QL), and multiple mutants were found to knock out serum response, including the chimera generated with Concertina.

While significant work has been done with the class-distinctive regions in $G\alpha_{12}$, there has been no experimentation with these regions in $G\alpha_{13}$. The purpose of this study is to generate similar mutants, including the Concertina chimera, in $G\alpha_{13}$ using the same determination of class-distinctive regions in order to compare $G\alpha_{12}$ and $G\alpha_{13}$ to one another and to the ancestral homolog, Concertina. SRE-luciferase encoding the firefly luciferase gene will be used as a benchmark to determine the activity of the generated mutants.

2. Methods

2.1 Construction of class-distinctive mutants:

Class-distinctive residues were found within the $G\alpha_{13}$ amino acid sequence and substituted for residues that were better conserved in other classes of G proteins using oligonucleotide-directed mutagenesis. When possible, restriction sites were either introduced or eliminated from the site of mutation in order to later determine the success of the procedure by isolating the resulting DNA, running a restriction digest, and then examining an agarose gel. When gels signified a successful mutation, the substitution was confirmed by DNA sequencing.

2.2 Construction of $G\alpha_{13}$/Concertina chimera:

A possible key region in the C-terminus of $G\alpha_{13}$ was replaced with the same region in Concertina using a triple sew method (Fig. 4). The three sections of sequence (two from $G\alpha_{13}$ and one from Concertina) were isolated using the polymerase chain reaction (PCR), with oligonucleotides designed to leave sticky ends. The sticky ends of the PCR products were then joined together via incubation, and success was gauged again by viewing band size on an agarose gel. Once the length appeared correct on the gel, the chimera was confirmed by DNA sequencing.

Serum Response Assay: Human embryonic kidney (HEK) cells were transfected, as previously described in Rasheed et al. 2013, with SRE-luciferase encoding the firefly luciferase gene, pRL-TK encoding the Renilla luciferase gene, and plasmids encoding $G\alpha_{12}$ or $G\alpha_{13}$ variants. After 32-40 hours, cell plates were removed from a 37° incubator, rinsed with PBS, and then removed from the wells using a passive lysis buffer. 5 µL of resulting sample were then mixed with 49 µL of LARII and read by a Glomax 20/20 Luminometer (Promega) resulting in the RLU1 value. Then, 49 µL of Stop&Glo buffer was added and again read to find the RLU2 value. The ratio between these two readings was then generated for each sample and used as a marker for protein production driven by $G\alpha_{13}$ mutant activity. Transfection with an empty vector with no G protein addition was used as a control.

2.3 Protein Binding Assay:

Myc-tagged $G\alpha_{12}$QL (activated $G\alpha_{12}$), Gα12WT (wildtype), $G\alpha_{13}$QL and $G\alpha_{13}$WT were tested on their affinity for binding to p115 and LARG. The p115 and LARG proteins were fused with heavy GST beads for quantification purposes. The myc-tagged proteins and GST-fusion proteins were mixed with HEDM buffer. 140 µL of the myc-tagged proteins were added to their respective interaction tubes, and then 100 µL of the GST-fusion/HEDM mixture was added in order for the interaction to take place. These were set in the refrigerator to mix on the Orbitron for 90 minutes at 120 rpm in order for complete binding interaction. The tubes were then centrifuged at 4000 rpm for 3 minutes at 4°C, removed, and all but 20 µL of the supernatant was pipetted off. 1 mL of HEDLM buffer was then added and the tubes were inverted to mix. This process was repeated (centrifugation, pipetting of supernatant, addition of HEDLM) two more times, and after the last pipetting of the supernatant, 15 µL of a 1:10 ratio of 4X and DTT were added to each sample. This was left to incubate in a 72°C water bath for 10 minutes. The resulting samples were then analyzed by western blot to compare binding affinity and a coomassie blue gel to confirm equal distribution of GST-fusion proteins between each interaction tube. Immunoblot results were quantified using a

Kodak GelLogic 100 imaging system equipped with Molecular Imaging 5.X software (Carestream Health, New Haven, CT) to calculate Gaussian fit for each protein band.

3. Results

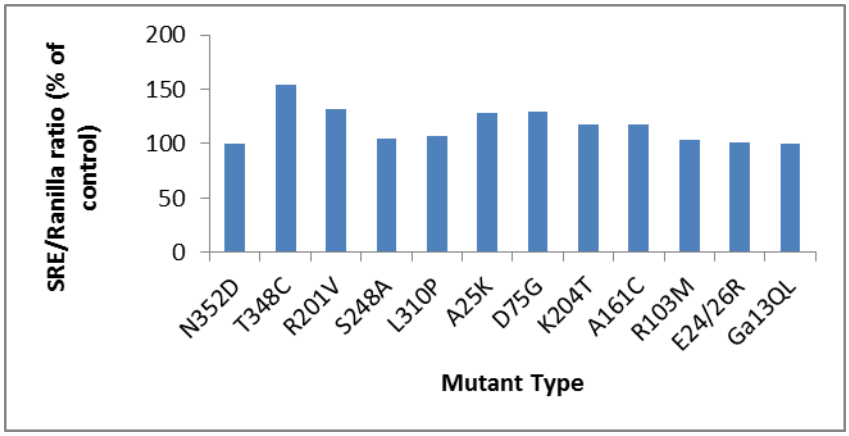

Figure 1. The percent comparison of each Gα13 mutant to the Gα13QL control of their SRE/Ranilla ratio.

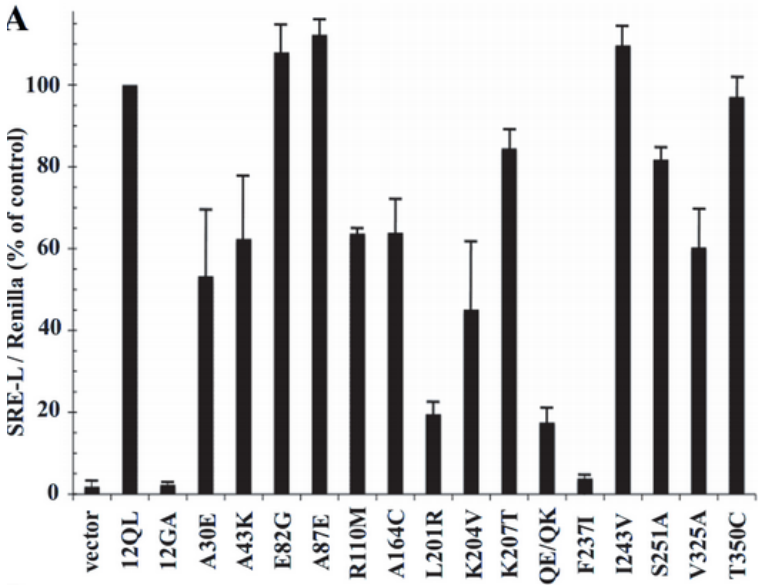

Figure 2. The percent comparison of each Gα12 mutant to the Gα12QL control of their SRE/Ranilla ratio.

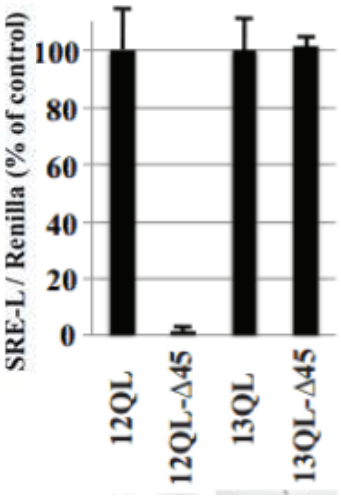

Figure 3: The percent comparison of each Concertina/G protein chimera to their QL control of their SRE/Ranilla ratio.

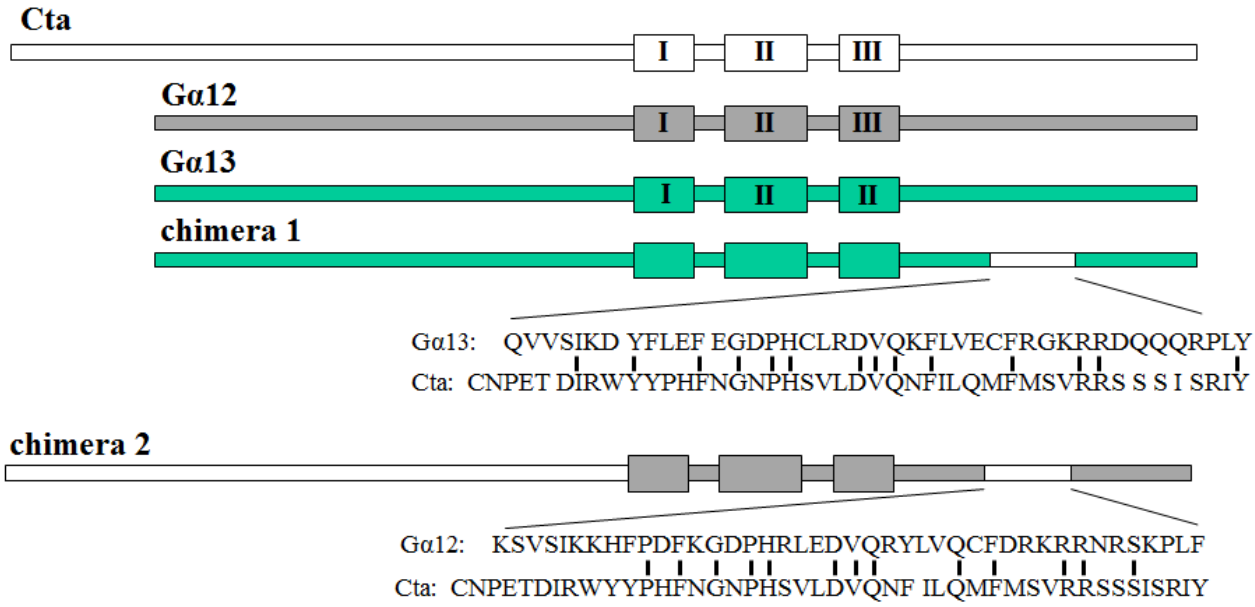

Figure 4: A schematic of the Gα13/Concertina (Cta) chimera, labeled as "chimera 1", and the Gα12/Concertina chimera, labeled as "chimera 2". Included are the three switch regions, and overlap between sequences is highlighted with a black bar. The same Cta sequence was inserted into both chimera 1 and 2 to replace the original Gα sequence.

Table 1: The Gaussian fit and ratio of fit to load for each mutant tested in the protein interaction study.

Mutant interaction	Gaussian Fit	Load Ratio
$G\alpha_{12}$QL/P115	8803.69	0.48607
$G\alpha_{12}$QL/LARG	5620.57	0.31032
$G\alpha_{12}$WT/P115	7292.25	0.35323
$G\alpha_{12}$WT/LARG	5145.74	0.29256
$G\alpha_{13}$QL/P115	20485.29	0.69301
$G\alpha_{13}$QL/LARG	29643.81	1
$G\alpha_{13}$WT/P115	313.36	0.01437
$G\alpha_{13}$WT/LARG	7135.68	0.32722

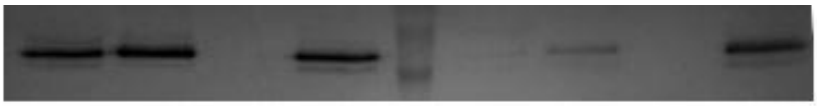

Figure 5: Pull down results of protein interaction study utilizing Ga13 mutants. From left to right: Ga13QL/P115, Ga13QL/LARG, Ga13QL/GST, Ga13QL load, ladder, Ga13WT/P115, Ga13WT/LARG, Ga13WT/GST, Ga13WT load.

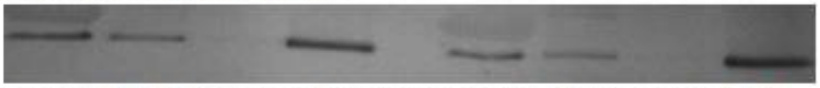

Figure 6: Pull down results of protein interaction study utilizing Ga12 mutants. From left to right: Ga12QL/P115, Ga132QL/LARG, Ga12QL/GST, Ga12QL load, blank, Ga12WT/P115, Ga12WT/LARG, Ga12WT/GST, Ga12WT load.

None of the eleven point mutants in $G\alpha_{13}$ had a detrimental effect on serum response activation compared to the $G\alpha_{13}$QL control (Fig. 1). Serum response activation between $G\alpha_{13}$ and Concertina also was not impacted compared to activated form of the protein, however, this chimeric mutation knocked out serum response in $G\alpha_{12}$ (Fig. 3). Fig. 2 shows that many of the point mutations engineered in $G\alpha_{12}$ caused significant impairment in SRE activation compared to the $G\alpha_{12}$QL control (Table1, Figs 5 and 6).

Data from protein interaction studies of wildtype (WT) versus activated (QL) mutants of $G\alpha_{12}$ and $G\alpha_{13}$ determined that $G\alpha_{12}$WT had a higher affinity for p115 and LARG RhoGEFs than did $G\alpha_{13}$WT. However, in a serum response assay, the SRE/Ranilla ratio dropped significantly from activated to wildtype form in $G\alpha_{12}$ (229.1compared to 166.8) and barely changed at all for $G\alpha_{13}$ (175.5 compared to 154.9) (Table 1).

4. Discussion

$G\alpha_{12}$ and $G\alpha_{13}$ are well characterized in the literature as stimulating a common pathway in cells and acting very similarly, and they have been described to utilize the same protein intermediates in serum response[3]. The data generated in this project suggest that there are very different regions within the amino acid sequence of these proteins that are critical in in activating this pathway. Comparing figures 1 and 2, it can be seen that none of the class-distinctive $G\alpha_{13}$ mutants knocked out serum response in the cell, but multiple class-distinctive $G\alpha_{12}$ mutants had potent disruptive effects. The same result happened in the chimeras incorporating the ancient Concertina C-terminal region into the $G\alpha_{12}$ and $G\alpha_{13}$ sequence. Levels were unchanged in $G\alpha_{13}$ yet were detrimental in $G\alpha_{12}$.

From all of these differences, we can determine that these two proteins utilize different regions of their structure in the serum response pathway, and perhaps different mechanisms altogether. Building from this, the activated and wildtype versions of $G\alpha_{12}$ and $G\alpha_{13}$ were tested for serum response, the wildtype Ga13 consistently triggered SRE activation at a high level, comparable to its mutationally activated form, whereas the wildtype Ga12 was typically much less potent in driving this pathway. Our recent pilot experiments have shown that the ability for wildtype (and presumably non-activated $G\alpha_{13}$) to drive SRE is not dependent on the serum used to grow the cells. The implication is that simply the overexpression of $G\alpha_{13}$ in a non-activated form is sufficient to drive serum response pathways resulting in activation of growth pathways such as proliferation and migration. If true, this could have major implications for tumor growth and metastasis in cancer types that overexpress this protein.

Preliminary protein-protein binding interaction studies were done to try to determine the differences in binding affinities for $G\alpha_{12}$ and $G\alpha_{13}$ to known downstream RhoGEFs and figures 5 and 6, as well as table 1, show that p115 and LARG do not show a higher binding affinity for wildtype $G\alpha_{13}$ over the activated version as expected. More RhoGEFs, such as PDZ, will be tested for the possibility of finding an effector that has a higher affinity for the wildtype form of $G\alpha_{13}$ over the wildtype $G\alpha_{12}$. This may give insight as to how $G\alpha_{13}$wildtpe can still drive serum response just has high as the activated form and what implications this may have physiologically.

5. Acknowledgements

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