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# Mutational Analysis of Non-Palmitoylated Cysteines in Gα12

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## Abstract

Gα12 and Gα13 acquire chemical information from outside the cell and move that information down pathways through guanine nucleotide binding. This pathway is essential for regulating functions including cell growth. Gα12 and Gα13 are proteins located on the cytoplasmic side of the cell membrane that bind guanine nucleotide exchange factors and other targets that regulate processes such as cell differentiation and cytoskeletal remodeling during cell division. Overexpressed Gα12 and Gα13 are implicated in various forms of cancer, including head and neck squamous cell carcinoma, so understanding their roles and regulatory mechanisms is crucial. My project examines the amino acid structure of the protein Gα12, specifically a set of cysteines for which it is not known whether the side chain is targeted for lipid modification. This event, termed palmitoylation, is essential for Gα12 to drive tumorigenic cell growth. Gα12 has a total of three cysteines, with previous research showing that the N-terminal cysteine (Cys-11) is an essential site of palmitoylation, a crucial component to signaling. My project was to engineer mutated variants of Gα12, targeting the other non-N-terminal cysteines to examine their role in tumorigenic signaling. The goal was to better understand the importance of these cysteines with the added benefit of creating mutated forms of Gα12, including a fully isolated N-terminal cysteine and a variant completely devoid of cysteines, to be used in future research. My initial experimental results suggest the cysteines beyond N-terminal Cys-11 are not essential for tumorigenic signaling by Gα12. Because the mechanistic details of Gα12 palmitoylation are yet to be understood, it is important to create these reagents for future experiments in which the palmitoylation state of Gα12 is probed directly.

## 1. Introduction

G proteins are heterotrimeric signal transduction proteins that localize to the inner surface of the plasma membrane in cells. They act as molecular switches that initiate signaling cascades

involved in many cellular processes, including inflammation, neurotransmission, and cellular regulation [3]. Gα12 is a monomer within the αβγ complex that makes up a full G protein and is conformationally bound to guanosine diphosphate (GDP) in its inactive state. Extracellular ligand binding to a membrane bound G protein coupled receptor (GPCR) changes the conformation of Gα12 allowing it to bind guanosine triphosphate (GTP) turning it to an "activated" state, which dissociates it from the complex and thus begins the signal cascade. G proteins are of critical importance in cell function and regulation as they signal to downstream enzymes like the Rho guanine nucleotide exchange factors (RhoGEF) which drive signaling for cellular processes like cytoskeletal rearrangement, cell division, and other cell regulatory processes [5]. The binding of GTP to Gα12 is what drives this signaling downstream, but signaling is regulated by cleaving a phosphate from its bound GTP back to GDP, which switches Gα12 back to an inactivated state. If a Gα12 monomer remains continuously bound to GTP they maintain activation, which can lead to oncogenic side effects. Gα subunits are considered oncogenes in that they have been implicated in multiple forms of cancer, including head and neck squamous cell carcinoma [3]. Irreversible GTP binding to Gα12 has been linked to higher mortality amongst patients with different types of cancers [2], so understanding the functional roles and structure of Gα12 is incredibly important toward cancer research. The protein sequence of Gα12 shows three cysteine amino acids at sequence positions 11, 239, and 332 within this 379 amino acid protein. Of these three cysteines, it has been shown that a lack of C11, through mutation to alanine, is detrimental to cellular signaling [4]. This is likely because C11 is the site of palmitoylation, which is the binding of palmitic acid to the cysteine and allows for Gα12 to be membrane bound. While we know that N-terminal C11 is critical to Gα12 signal function, we are unsure of the role, if any, of C239 and C332 in Gα12 signaling function. Considering how crucial C11 is to an oncogenic signaling pathway, it is important to understand if C239 or C332 are also involved in Gα12 signaling and to what extent. The goal of this project was to engineer sequence homologs of Gα12 that take advantage of the non-polar amino acid alanine to replace the individual cysteines in the amino acid sequence. The cysteines we targeted were C239 and C332 individually with the additional goal of creating a double mutant of C239 and C332 to alanine. These mutant forms of Gα12 were tested for their ability to drive oncogenic signaling in cultured human cells. Furthermore, a secondary goal of creating various knockout mutants, including a mutant where all cysteines are missing, is being carried out and could be useful for further research.

# 2. Methods and Materials

## 2.1 Creation of Cysteine Lacking Gα12 mutants using PCR

The first step was to design forward and reverse oligonucleotides for mutagenesis of Gα12, which was done by replacing the cysteine residues with alanine using PCR. Gα12 mutants with an existing mutation of cysteine 332 to alanine (C332A) as well as a myc-tagged Gα12 (12mq1) sequence were provided by Dr. Ted Meigs. We designed forward and reverse oligonucleotides that were 32 base pairs in length, which were ordered from Eurofins Genomics. They were centrifuged for 5 minutes at 1000 x gravity then diluted with 200 µL of Qiagen EB buffer and vortexed for 30 seconds. After sitting for 20 minutes, they were vortexed again then centrifuged for a few seconds at 16,000 x g. We then used a GeneSys spectrophotometer at 260 nm wavelength at 100x dilution with deionized water to each oligo to measure absorbance. This was used to calculate and create 5

µM stocks of each oligo. Next, 5 µM stocks of the primers CMV 635 FWD and WeimbsBGH-Rv were made which were to be used for PCR. Forward PCR stocks of the cysteine 332 and 229 knockout (C332F) included 10 µL of 2X Q5 hotstart master mix, 2 µL of forward oligo, 2 µL of WeimbsBGH-Rv, 1 µL of C332A and 5 µL of water while reverse stocks (C332R) included 10 µL of master mix, 2 µL of reverse oligo, 2 µL of CMV.635.fwd, 1 µL of C332A and 5 µL of water. Forward PCR stocks of the single c239 knockout (12q1A) included 10 µL of master mix, 2 µL of forward oligo, 2 µL of WEIMBSrv, 1 µL of 12mq1, and 5 µL of water while reverse stocks (12mq1B) included 10 µL of master mix, 2 µL of reverse oligo, 2 µL of WEIMBSrv, 1 µL of 12mq1, and 5 µL of water. These PCR products were then isolated using gel electrophoresis and ran at 110 Volts for one hour. The necessary bands were then cut from the gel and DNA was purified using the Promega Wizard SV kit.

## 2.2 Digestion and Ligation

In order to proceed to transforming our DNA constructs into *Escherichia coli*, we first prepared restriction digests of each PCR product as well as digestion of pcDNA3.1 plasmid for the digests to be ligated into. The next step was to create digest "sticky ends" using NheI-HF and KpnI-HF restriction enzymes. Next, 20 µL of each PCR product was added to 2 µL of 10X Cutsmart buffer, 0.5 µL of NheI-HF and 0.5 µL of KpnI-HF. We wanted 3.0 µg of DNA from our plasmid digest so we calculated that 3 µL of pcDNA3.1 would be added to 14 µL of water, 2 µL of 10X Cutsmart buffer and 0.5 µL of each restriction enzyme. These mixtures were mixed then centrifuged before being added to a 37°C water bath for four hours. The digests were then isolated using gel electrophoresis and ran at 110 Volts for one hour. Bands were then cut from the gel and DNA was purified using the Promega Wizard SV kit. Concentration of DNA was determined using a Nanodrop spectrophotometer at 260 nm wavelength. For ligation, 4.5 µL of pcDNA3.1 plasmid were used to generate 200 ng of plasmid. Two ligation stocks were made using 12.5 µL of each insert mixed with 4.5 µL of pcDNA3.1, 2 µL of 10x ligase buffer, and 0.7 µL of DNA ligase. These mixtures were left to incubate for one hour at room temperature.

## 2.3 Transformation into *Escherichia coli*

Bacterial cells are known to respond to extreme stresses by taking up extracellular DNA from their environment when induced by chemical treatment, so this methodology was used to have treated *Escherichia coli* uptake our newly manufactured plasmids to produce a large number of them. JM109 *Escherichia coli* cells were retrieved from a -80°C freezer and put on ice to thaw. JM109 cells were added to three separate Falcon tubes in 15 µL increments while being kept on ice. 2 µL of each ligation were then added to two of the three tubes and none were added to the third tube as a control These were placed back on ice and left to sit for 30 minutes. The mixtures were then heat shocked in a 42°C water bath for 45 seconds before immediately being placed back on ice. 0.4 mL of SOC medium was added to each tube which were then placed in a 37°C shaking incubator set to 230 rpm for one hour. Maintaining a sterile workspace, these tubes were then transferred to separate micro centrifuge tubes then centrifuged at 9000 xg for three minutes. The supernatant was removed leaving the pellet of cells and approximately 100 µL of media then titrated to resuspend the pellet. This cell mix was then moved onto LB-Ampicillin plates (100 µg/mL) and spread using sterile beads for one minute, then rest for five minutes before being moved into a 37°C incubator for 16 hours.

## 2.4 Incubation

The next step was to grow liquid cultures of workable colonies from the LB-Ampicillin plates. Using a 10-mL stock of LB-ampicillin with a concentration of 75 µg/mL ampicillin in LB liquid, we pipetted 2 µL of the stock into 5 separate Falcon tubes, two dedicated to a separate colony from each plate and one control. Plates with colonies were warmed to room temperature for 15 minutes before non-overlapping colonies were chosen to be picked using sterile toothpicks and then added to each Falcon tube. The control tube involved exposing a sterile toothpick to the air by swishing it around before placing it in a tube. The toothpicks stayed in the tubes while they were incubated at 37°C for 14 hours and shaking at 230 rpm. After incubation, 1.4 mL was taken from each Falcon tube and pipetted into a fresh microcentrifuge tube before being spun at 9000 x gravity for five minutes. Liquid supernatant was extracted from each tube as close to the cell pellet as possible, without disturbing the pellet.

## 2.5 Plasmid Purification

Using a Qiagen purification kit, 250 µL of P1 buffer was added to each tube straight onto the pellet of cells then titrated until the pellet was no longer visible. Over the span of five minutes, each tube was given 250 µL of P2 lysis buffer one at a time, each time inverting to mix before starting the next tube. After exactly five minutes, 350 µL of N3 neutralizing buffer was added to each tube, once again individually one at a time, before being inverted to mix and then moving to the next tube. After ensuring that the blue color of the lysis buffer was no longer visible, indicating a return to neutral pH, the tubes were centrifuged for 10 minutes at 16000 x gravity. The supernatant from each tube was then added to its own Qiagen affinity column placed in a catch tube and then left to sit for two minutes before being centrifuged at 16000 x gravity for one minute. After discarding the contents of the catch tube, 0.5 mL of PB buffer was added to each column and was left to sit for one minute before being centrifuged at 16000 x gravity for one minute. The contents of the catch tube were discarded and 0.75 mL of PE wash buffer were added to each column, left to stand for two minutes, then centrifuged again at 16000 x gravity for one minute. Each catch tube was then discarded and the columns were added to microcentrifuge tubes with the lids removed then centrifuged at 16000 x gravity for two minutes without the normal plastic lid over the centrifuge unit (this is to completely remove ethanol from the final DNA mix). The cap-less microcentrifuge tubes were then discarded and the column was then added to a fresh microcentrifuge tube with the lid left on. After centrifugation, 50 µL of EB DNA elution buffer (Qiagen, Germantown, MD) was then added directly into the center of each column, left to stand for three minutes and then centrifuged for one minute at 16000 x gravity. 2 µL of each sample were then measured on the nanodrop spectrometer to get the concentration of DNA and the 260/280 ratio in each sample.

## 2.6 Prepping for sequencing

To confirm correctly mutated cDNA, samples were sequenced by an external biotechnology company (Azenta, Research Triangle Park, NC). Before sequence prep, viable colonies were taken and streaked on fresh LB-ampicillin plates for preservation in case sequencing needed to be revisited. Four 20 µL working stocks were created at 70 ng/µL concentration and diluted with EB elution buffer before being divided into four 10 µL PCR tubes, two for each plasmid to be read forward and backward. These tubes were then shipped to Azenta to be sequenced.

## 2.7 Transfection of Plasmids into Human Kidney Cells

To encode the Gα12 variants engineered in this project, plasmids were transfected into human kidney cells. Six working stocks were created at 20 ng/μL concentration of DNA using EB elution buffer then these were distributed at 5 μL increments to 12 tubes labeled 1 through 12, with 1 through 6 and 7 through 12 being duplicate stocks. Next, we created a 130 μL master mix containing the reporter plasmids SRE luciferase (200 ng/μL) and renilla (pRL-TK) (20 ng/μL) and added 10 μL of this mix to each of the 12 tubes. With sterility in mind, under a hood, 70 μL of Eagles medium and 2 μL of Polyethylenimine (PEI) at a concentration of 2.0 mg/mL were added to each tube, titrated and flicked to mix then left to sit for 15 minutes. Using a drip then swirl method, each tube of our constructed DNA plasmids were added to its own well of human kidney cells dropwise. The wells of cells were then placed in an incubator for 32 hours.

| Stock | C332A, 12mql, myc | C239A, C332A,12mql , myc (TM2, C332A) | C239A, 12mql, myc (TM4, 12mq) | Gα12 (12mql ONLY) | pcDNA (no Ga12 protein) | C11A, 12mql, myc |
|---|---|---|---|---|---|---|
| Concentration | 1430 ng/μL | 245 ng/μL | 217.5 ng/ μL | 790 ng/ μL | 838.2 ng/μL | 938 ng/μL |
| EB buff | 70.5 μL | 33.8 μL | 29.6 μL | 38.5 μL | 40.9 μL | 45.9 μL |
| Total volume: | 71.5 μL | 36.8 μL | 32.6 μL | 39.5 μL | 41.9 μL | 46.9 μL |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C11A | Ga12 | pcDNA | C239A | C239A, C332A | C332A | C11A | Ga12 | pcDNA | C239A | C239A, C332A | C332A |

**Table 1.** shows the working stocks for the C332A mutation DNA (C332A, 12mql, myc), the C239A & C332A mutation DNA (C239A, C332A, 12mql, myc), the C239 mutant DNA (C239A, 12mql, myc). Controls included unmutated Gα12 DNA (Gα12), pcDNA 3.1 with no insert (pcDNA), and C11A which was provided by Dr. Meigs and had been tested in previous research to have little to no signaling (C11A, 12mql, myc). Concentration designates the concentration of DNA per stock in ng/μL. EB buff is the total volume of EB elution buffer added to each stock measured in μL. The total volume is the target volume in μL. The bottom figure shows the order in which the tubes were filled before being added to the wells of human kidney cells by number designation.

## 2.8 Cell Protein Purification

We created a stock of 3.5 mL of 1X passive lysis buffer. Next, we took the incubated wells of cells and discarded any liquid, leaving just the cells then replaced the liquid with 1X PBS buffer into each well. The 1X PBS was then removed and replaced with 250 μL (to each well) of the 1X passive lysis buffer prepared earlier. The wells were then placed in a shaker set to 120 rpm for 25 minutes. A master mix was then created using 350 μL of 4X buffer and 35 μL of 1M DTT. 20 μL of master mix was added to 12 microcentrifuge tubes labeled “passive.” The wells of cells were then removed from the shaker and pipetted into a corresponding numbered microcentrifuge tube. The cell tubes

were then centrifuged at 16000 xg for 30 seconds before 40 µL of supernatant was removed from each tube and added to its corresponding “passive” tube then mixed. The tubes labeled “passive” 1-12 were then placed in a 72°C water bath for 10 minutes. The 12 tubes not labeled as “passive” containing cell pellets were then placed into the -80°C freezer while the 12 tubes labeled “passive” 1-12 were placed in a regular freezer.

## 2.9 Gα12 Signaling

The 12 “passive” samples were retrieved from the -80°C freezer, thawed, then centrifuged. We then made a stock solution consisting of 350 µL of Stop and Glo buffer and 7 µL of Stop and Glo 50x substrate which was stored in an amber vial. 24.5 µL of LAR II stored in the -80°C freezer was then added to 12 microcentrifuge tubes. Now one at a time for each sample, 2.5 µL of the labeled supernatant was then added to a LAR II tube before being mixed and then read using a Glomax 20/20 Luminometer. After the first reading, the tube was removed and given 24.5 µL of the Stop and Glo solution from the amber vial then read again using the luminometer. The two values were used to create a ratio. This process was repeated for each of the 12 samples.

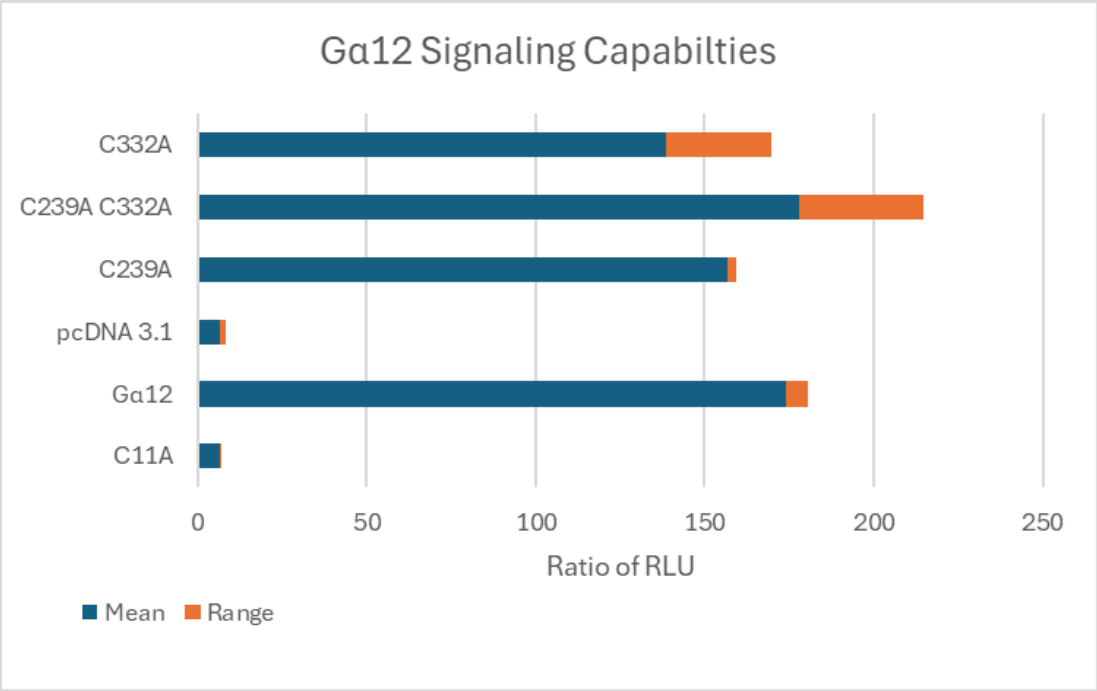
# 3. Results

## 3.1 Mutation products

PCR, digestion, and ligation yielded usable plasmids of our sought after knockout mutations of C239A and C239A + C332A mutants of Gα12. Sequencing results did reveal that the C239A mutant had 100% identity to regular Gα12 excluding our cysteine mutation, but the double mutation (C239A + C332A) had an additional mutation at the 5th residue valine to a leucine. This unwanted mutation was found after the luciferase assay had been performed.

## 3.2 Luciferase assay

The benefit of the luciferase enzyme is its ability to bind our substrate and allow us to measure expression rates based on luminosity, which is stated in Real Light Units (RLU). As expected, Gα12 at its 11th positioned cysteine, when mutated to alanine, was detrimental to its signaling capabilities (Figure 1: 6.62, 6.25 RLU). Mutated Gα12 at its 239 cysteine to alanine showed little to no impact on signaling (Figure 1: 157, 155 RLU) as well as the cysteine 332 to alanine mutant (Figure 1: 154, 122 RLU). The double knockout of C239 and C332 mutated to alanine also showed little to no impact on signaling (Figure 1: 196, 159 RLU). For reference, these mutated samples were run alongside a control of regular, unmutated Gα12 which, as expected, showed normal signaling (Figure 1: 170, 177 RLU). A control was also tested with plasmid pcDNA with no G protein insert, meaning it lacked the ability to signal, which showed little to no signaling (Figure 1: 7.43, 5.83 RLU).




| | 1<br>C11A | 2<br>Ga12 | 3<br>pcDNA | 4<br>C239A | 5<br>C239A<br>C332A | 6<br>C332A | 7<br>C11A | 8<br>Ga12 | 9<br>pcDNA | 10<br>C239A | 11<br>C239A<br>C332A | 12<br>C332A |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| RLU 1 | 18,181 | 372,766 | 25,318 | 379,336 | 431,974 | 401,987 | 15,996 | 477,696 | 28,415 | 398,980 | 370,301 | 508,895 |
| RLU 2 | 2,744 | 2,183 | 3,407 | 2,401 | 2,199 | 2,608 | 2,561 | 2,694 | 4,872 | 2,568 | 2,317 | 4,152 |
| Ratio | 6.62 | 170.73 | 7.430 | 157.963 | 196.409 | 154.106 | 6.245 | 177.283 | 5.831 | 155.34 | 159.792 | 122.558 |

**Figure 1**. Shows graphed results of Gα12's signaling capabilities read on the Glomax 20/20 Luminometer using the luminescent Luciferase firefly enzyme. The bar chart shows the amount of signaling represented by a ratio of RLU 1 to RLU 2. RLU 1 is the reading before the Stop and Glo solution was added. This ratio gives a good representation signaling capability. The chart also shows a mean for each stock as each was measured twice on the luminometer. To show the difference between the two measurements, the range between values is also represented on the bar chart.

**Table 2.** Shows the data collected from the initial luciferase luminometer reading. The numbered stocks 1-12 also represent the numerical order in which they were measured in the luminometer.

# 4. Discussion

Palmitoylation of Gα12 involves the enzymatic, covalent attachment of a single palmitic acid, a common fatty acid found both in and out of mammalian cells, to the N-terminal cysteine C11 which is responsible for adhering the subunit to the cell membrane. The overall goal of this research was to see whether the non-N-terminal cysteines play any role in Gα12 signaling for cancerous cell growth. It seems that, based on our results, Gα12's ability to palmitoylate is directly

related to its N terminal cysteine exclusively. This insinuates that there is a link between this membrane bound palmitoylation and Gα12's ability to signal, although the fact that it is specifically "membrane bound" could be irrelevant as palmitoylation may serve another function which has been linked to protein folding, protein stability, and, most importantly, interactions with other proteins [1]. The next logical step forward would be to create mutants that have all cysteines mutated to alanine to not only test this mutant's ability to signal, but also to use in further research into non-palmitoylated Gα12.

Before beginning the luciferase assay to test signaling capabilities, we sent our engineered plasmids to Genewiz to be sequenced to ensure that our sequences were correct. We were sent back a sequence that correctly matched Gα12 for the C239A mutant but harbored a valine to leucine mutation close to the N-terminus on the C239A + C332A mutant. This mutation likely had no direct impact on signaling capabilities during our luciferase assay, but we cannot be sure, so it is essential to fix this mutation to substantiate our results. Work is underway to cross splice out this mutation with a known sequence from our mutants using the restriction enzyme BglII to cleave the DNA at positions flanking the unwanted mutation. Since only one restriction enzyme is being used, careful attention has been given to use a shrimp alkaline phosphatase (RSAP) to prevent pcDNA3.1 from ligating to itself and ensuring our insert is ligated instead. This research is ongoing and has not yielded any products as of yet.

It cannot be overstated the importance of Gα12 in relation to cancer research. From 2010 - 2019, 14 different types of cancers have increased in diagnosis amongst Americans aged 15 - 49 [5]. Understanding the functional pathways and structural components of G proteins or its associations could hold answers into potential pharmaceutical practices to combat uncontrolled cell proliferation.

# 5. Acknowledgements

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