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Gα12-to-Concertina Mutagenesis to Identify Structural Determinants that Regulate Cell Growth

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Abstract

Guanine nucleotide binding proteins of the G12/13 subfamily regulate a number of cellular processes that have the ability to become aberrant in cancers. Within this subfamily, Gα12 in particular has been found to provide a key function in the serum response pathway (SRF), leading to stimulation of the transcriptional activator SRF in the nucleus of a cell. Concertina, a homolog of Gα12 in *Drosophila,* does not participate in activation of the serum response pathway when expressed in human cells. Previous work in Dr. Meigs' lab found a 42-amino acid region near the C-terminus of Gα12 to be necessary for activation of this pathway. To better understand the cellular mechanisms triggered by this region of Gα12, specific amino acids within this region were changed to their Concertina counterparts. This process was performed using molecular biological techniques, and several mutants of Gα12 were generated and confirmed by DNA sequence analysis. These mutants are being tested for their ability to drive transcriptional activation through SRF in cultured human cells. Understanding mechanisms that alter Gα12 function in cell proliferation may ultimately prove to be viable targets for antineoplastic drugs.

1. Introduction

Many effector molecules within a eukaryotic ell are stimulated by extracellular signals from G proteins[1]. G proteins are classified by one of their two functional units, the α-subunit. The α-subunits function as GTPases and are able to exist in two distinct states due to guanine nucleotide exchange and hydrolysis[2]. The α-subunit subfamilies (Gs, Gi, Gq and G12) are classified according to the primary sequences of their α-subunits[1]. The G12 subfamily consists of Gα12 and Gα13 and is fairly distinct among heterotrimeric G proteins in several ways. First, this subfamily regulates many cellular processes such as cell growth, migration, cell-cell adhesion and cell-substrate adhesion. All of these processes have the ability to become deviant in cancers indicating Gα12 and Gα13 play pivotal roles in cancer invasion and metastasis[3]. Also, Gα12 and Gα13 are unique in their ability to exert oncogenic effects in cells when overexpressed in the cell. Gα12, in particular, was found to exhibit the ability to stimulate focus formation in NIH3T3 mouse fibroblasts when overexpressed in wild-type form revealing its identity as a transforming oncogene[3]. Lastly, Gα12 interacts with many more target proteins than do the other Gα subunits[4]. Therefore, many Gα12 interactions are yet to be fully understood.

There are numerous Gα12 homologs in evolutionarily divergent taxa. These homologs, while not identical to Gα12, contain many conserved amino acid regions. One such homolog, Concertina (Cta), is found in *Drosophila melanogaster* and is highly similar to Gα12. The two proteins have a 54% amino acid identity, which is high considering their vast evolutionary distance. In previous studies, it was found that Gα12 was able to activate the serum response pathway while Concertina was not. Also, a 42-amino acid region near the C-terminus of Gα12 shown in Figure 1 was found to be essential for SRF activation[5]. This is particularly interesting as SRF controls transcription of genes required for entry into the cell cycle.

It is important to determine which of these 42-amino acids are necessary and sufficient for serum response factor activation and subsequent cell proliferation. This study sought to engineer Gα12 to Concertina amino acid substitutions to identify which specific mutations disrupt Gα12 function in activating SRF in cultured human cells. In this paper, strategies for Gα12 mutagenesis and strategies to determine the presence of the engineered mutant are discussed. The processes and mutants generated in this study can be used in further studies to determine the importance of the Gα12 region replaced by Concertina amino acids in cell proliferation.

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           230        240        250        260        270        280
Ga12   VDVGGQRSQRQKWFQCFDG-ITSILFMVSSSEYDQVLMEDRRTNRLVESMNIFETIVNNK
       ::::::::.::::: .::::. .::::.:::::::::::: :::.:::: :: :::.::::: 
Cta    VDVGGQRTQRQKWTRCFDSSVTSIIFLVSSSEFDQVLAEDRKTNRLEESKNIFDTIVNNA
       300        310        320        330        340        350

            290        300          310        320        330        340
Ga12   LFFNVSIILFLNKMDLLVEKVKS--VSIKKHFPDFKGDPHRLEDVQRYLVQCFDRKRRNR
        : ..::::::::: ::: .::. .  ..:. ..: :.:.:: . ::: ...: :   ::. 
Cta    TFKGISIILFLNKTDLLEQKVCNPETDIRWYYPHFNGNPHSVLDVQNFILQMFMSVRRSS
       360        370        380        390        400        410

             350        360        370
Ga12   S-KPLFHHFTTAIDTENIRFVFHAVKDTILQENLKDIMLQ
       : . ..:::::::::.::  ::..:::::::.::. .:::
Cta    SISRIYHHFTTAIDTRNINVVFNSVKDTILQRNLNALMLQ
       420        430        440        450
```

Figure 1. Comparison of Gα12 and Concertina C-terminal domains. The underlined region is the C-terminal domain and the purple highlighted section constitutes the 42-amino acid region necessary for SRF activation in Gα12.

2. Methods

A constitutively activated form of Gα12 (Myc-Gα12^{QL}) was used throughout the entire project. A cDNA encoding myc-Gα12^{QL} was previously inserted by other lab members into the vector pcDNA 3.1 (Invitrogen) containing an ampicillin resistance sequence (Figure 2).

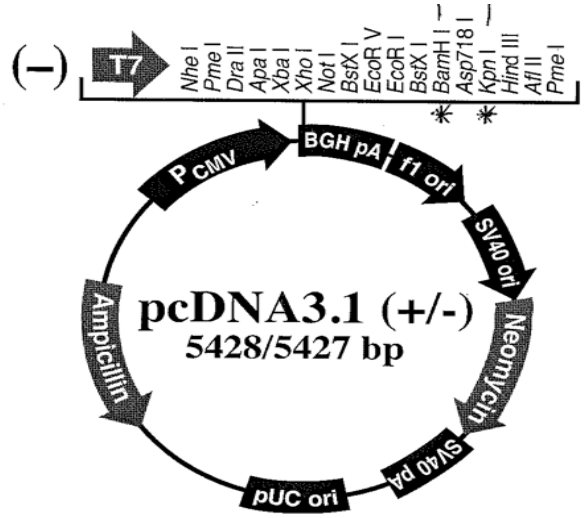


Figure 2. pcDNA3.1 (Invitrogen) plasmid where Gα12 candidate mutants were inserted

2.1 PCR Sewing

A two-step PCR process, was performed to obtain a number of Gα12-to-Concertina mutants. For each mutagenesis strategy, two oligonucleotides were engineered to contain the desired Concertina residue substitutions as well as an overlap of at least 24 base-pairs with native Gα12 sequence. Furthermore, each sew oligonucleotide pair was designed to exhibit complementation of 18 base-pairs. End forward and end reverse oligonucleotides complementary to pcDNA 3.1 sequence outside of the Gα12 cDNA were used to amplify the remaining Gα12 sequence upstream and downstream of the substitution. PCR was performed to yield two amplified regions with an overlap of 18 base-pairs. These regions were loaded into an agarose gel electrophoresis apparatus with 4 μL 6X loading dye and allowed to run for approximately 1 hour at 120 V. The gel was observed on the Kodak gel documentation system. The bands of correct predicted length were cut and extracted from the agarose gel using the Promega Wizard SV column and analyzed on the Nanodrop spectrophotometer. The two regions were then combined and subjected to another round of PCR containing only the end forward and end reverse oligonucleotides in order to amplify the entire candidate mutant Gα12 sequence. The PCR product of this reaction was then run on an agarose gel electrophoresis apparatus and analyzed as previously stated.

2.2 Candidate Mutant Analysis

To confirm the presence of desired mutations in Gα12 sew PCR products, a series of reactions were implemented.

2.2.1 ligation of candidate mutant cDNA of Gα12 into pcDNA 3.1 plasmid

Restriction digests were used to ligate each of the candidate Gα12 mutants into the original plasmid. Enzymes were chosen based on their ability to cut the plasmid only once each while also only cutting the insert once each. Restriction enzymes used in these experiments were a combination of NotI and KpnI or EcoRI and KpnI. All of the enzymes used in this study were the HF (high fidelity) forms. The sew PCR product digestion reactions each contained 3 μL of Cutsmart buffer (New England Biolabs, 240 County Road, Ipswich, MA 01938), μL of each restriction enzyme and 25 μL of sew PCR product. The plasmid digestion reaction consisted of 3 μL Cutsmart buffer, 1 μL of each restriction enzyme, 3 μg of plasmid, and an amount of deionized H_2O to bring the reaction to 30 μL. These reactions were placed in a 37°C water bath for 90 minutes. After 30 minutes in the water bath, 2 μL of CIP was added to the plasmid reaction to prevent the cut plasmid from rejoining, and the reaction was placed back into the water bath for the remaining 60 minutes.

After 90 minutes, the samples were loaded into an agarose gel electrophoresis apparatus with 6 μL 6X loading dye and allowed to run at 120 V for approximately 1 hour. The PCR samples were cut and extracted from the gel using the Promega Wizard SV column after being observed in the Kodak gel documentation system. The plasmid band at approximately 5,500 bp was carved from the gel and extracted using the same technique. All extracts were then analyzed on the Nanodrop spectrophotometer to determine DNA concentration.

Ligation reactions were set up for each sample in microfuge tubes. To each tube 150 ng of plasmid, 2 μL of 10X buffer with ATP, 1μL of T4 DNA ligase, 2-6 μL of each candidate mutant Gα12 cDNA and deionized water to total 20 μL was added. These tubes were left at room temperature for 1 hour and placed in a 4°C refrigerator for 1 hour. Afterward, the ligations were run on an agarose gel electrophoresis apparatus with 4 μL 6X loading dye for 1 hour at 120 V. The gels were then analyzed using the Kodak gel documentation system. Samples were stored in a freezer until bacterial transformation was performed.

2.2.2 bacterial transformation

An aliquot of JM109 cells was obtained from an -80°C freezer and placed into a bucket containing ice and a small amount of water to thaw. One 14-mL snap-cap, round bottom Falcon tube for each sample was placed into a bucket of ice with no water. JM109 cells were flicked gently and separated evenly into each Falcon tube kept on ice. Excluding the control tube, 1 μL of each ligation was added to directly to the puddle of cells found in a Falcon tube and flicked gently. These tubes were kept on ice for 30 minutes. Falcon tubes were then heat-shocked in a 42°C water bath for 45 seconds and quickly placed into ice. The tubes were incubated on ice for 2 minutes. 0.7 mL of

SOC medium was then pipetted into each Falcon tube and placed in a 37°C incubator shaking at 230 rpm for 1 hour. Tubes were removed from the incubator, and the content of each Falcon tube was pipetted into a sterile microfuge tube. These tubes were centrifuged at 9,000g for 3 minutes. All except 100 μL supernatant from each tube was discarded into Clorox waste. The cell pellet was resuspended by trituration. LB-ampicillin plates were obtained for each sample. Approximately 30 sterile glass beads were poured onto each plate, and the contents of each tube was pipetted directly onto the center of the plate. The lid was replaced and beads were slid around the plate to ensure full contact of the plate surface. Beads were discarded into the Clorox waste after 5 minutes. The plates were placed lid-down into a 37°C incubator for approximately 12 hours. Afterward, each plate was observed for colonies.

2.2.3 colony inoculation

After confirmation of colonies, an LB-ampicillin broth containing an ampicillin concentration of 75 μg/mL was prepared. 2 mL of the mixture was added to a new sterile snap-cap, round bottom Falcon tube for each selected colony. Colonies were selected and marked from each plate and touched with a sterile toothpick that was then submerged in the LB-ampicillin solution. A sterile toothpick that had no contact with a colony was placed in an LB-ampicillin solution Falcon tube to create a control. These tubes were placed in a 37°C shaking incubator for 12 hours. After observing no growth in the control tube, 1.2 mL from each Falcon tube was pipetted into a sterile microfuge tube and centrifuged at 9,000g for 3 minutes. The supernatant was discarded and cell pellets were frozen until plasmid elucidation. The LB-ampicillin plates were wrapped with parafilm and stored in a lab refrigerator.

2.2.4 plasmid purification

A Qiagen mini-prep kit was obtained to perform plasmid isolation from cell pellets. 250 μL of P1 buffer was added to each cell pellet and triturated 15 times until the pellet was no longer visible. 250 μL of P2 buffer was quickly added to one tube of suspended bacterial cells and inverted twenty times resulting in blue color. This process was repeated for each tube. When the first tube had incubated for 5 minutes, 350 μL of N3 was added, and the tube was inverted 20 times removing the blue color. This treatment was applied to each sample. The tubes were then centrifuged for 10 minutes at 16,000g. Each supernatant was pipetted into an affinity column positioned inside a capless round-bottom tube. These assemblies were centrifuged at 16,000g for 1 minute. The catch tube was emptied of flow-through liquid after centrifugation in all steps prior to DNA elution. 0.5 mL of PB buffer was pipetted into each column and centrifuged for 1 minute at 16,000g. 0.75 mL of PE buffer was pipetted into each column and left standing upright for 1 minute. The columns were then centrifuged for 1 minute at 16,000g, and this step was repeated a second time. Each column was then placed into a decapitated microfuge catch tube and centrifuged for 1 minute at 16,000g with the internal plastic lid left off. The columns were then placed into sterile, lidded microfuge tubes, and 50 μL of EB buffer was pipetted directly onto the center of the column. The tube was left upright for 5 minutes and afterward centrifuged for 1 minute at 16,000g with the plastid lid on. These samples were stored in a freezer until restriction digest reactions were preformed.

2.2.5 diagnostic restriction digests

Restriction digest were performed on all candidate mutants. For each candidate, a digest reaction tube was created containing 2.2 μL Cutsmart buffer (New England Biolabs, 240 County Road, Ipswich, MA 01938), 0.5 μL of each restriction enzyme, 5 μL of the candidate mutant sample, and 13.3 μL of deionized H_2O to total 20 μL. The digests were placed in a 37°C water bath for 90 minutes. Afterward, the samples were loaded onto an agarose gel electrophoresis documentation system with 4 μL 6X loading dye and allowed to run for approximately 1 hour at 120 V. The gel was then analyzed on the Kodak gel documentation system. Samples exhibiting bands of predicted fragment length were sent to Davis Sequencing (Davis, CA) for sequencing. The sequencing results were analyzed to confirm correct Gα12-to-Cta mutagenesis.

2.3 Sequence Analysis of Previously Created Mutant Candidates

Four mutant candidates were created by the Fall 2013 BIOL344 class by oligonucleotide directed mutagenesis. These sequences were obtained after being sent to Davis Sequencing (Davis, CA) by Dr. Meigs. The ExPASy

Translate tool and LALIGN pairwise alignment tool were used to compare candidate mutant amino acid sequences with that of native Gα12.

3. Results

3.1 Sew PCR

Oligonucleotides created to contain Cta substitutions used in sew PCR part 1 are shown in Figure 3 for two mutants. Gel electrophoresis of the first round of sew PCR for the same two mutants produced fragments of predicted length (Figure 4). Figure 5 shows the successful ligation of the two sew PCR products using only end oligonucleotides.

sewing PCR oligonucleotides
K^{310}HF to WYY mutant
5'-AAGTGGTATTATCCAGATTTCAAGGGCGACCCG-3' (sew forward)
5'-ATCTGGATAATACCACTTCTTAATGCTCACAGACTT-3' (sew reverse)

D^{334} to M mutant
5'-GTGCTTCATGAGGAAGCGCAGGAACCGCAGC-3' (sew forward)
5'-CGCTTCCTCATGAAGCACTGCACCAGGTAGCG-3' (sew reverse)

Figure 3. The oligonucleotides used for sewing PCR with the mutation sequence of Cta to be inserted highlighted blue. The underlined region indicates the region of oligonucleotide complementation.

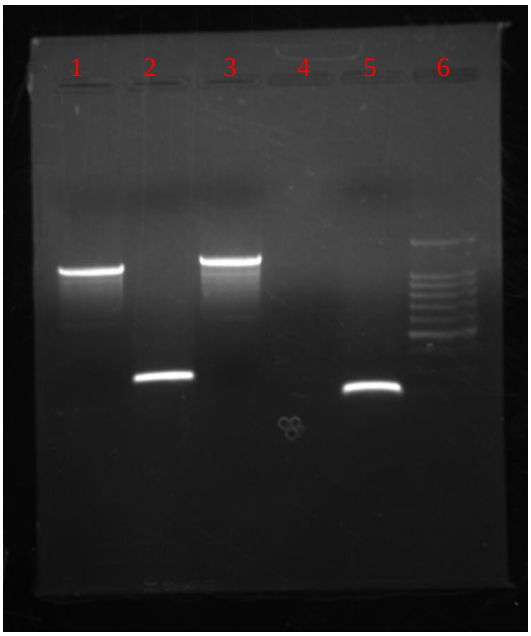


Figure 4. Gel image of bands produced after the first round of sew PCR yielding two fragments per mutant candidate. Lanes 1 and 2 correspond to KHF to WYY, and lanes 3 and 5 correspond to D to M. Lane 6 contains the 100 bp ladder.

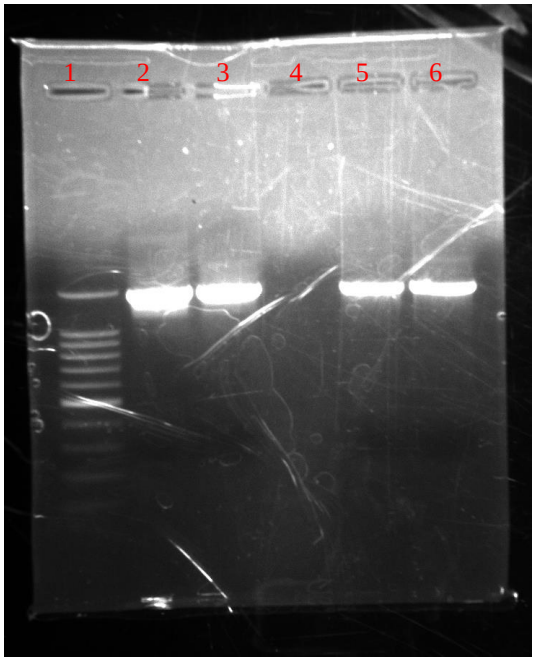
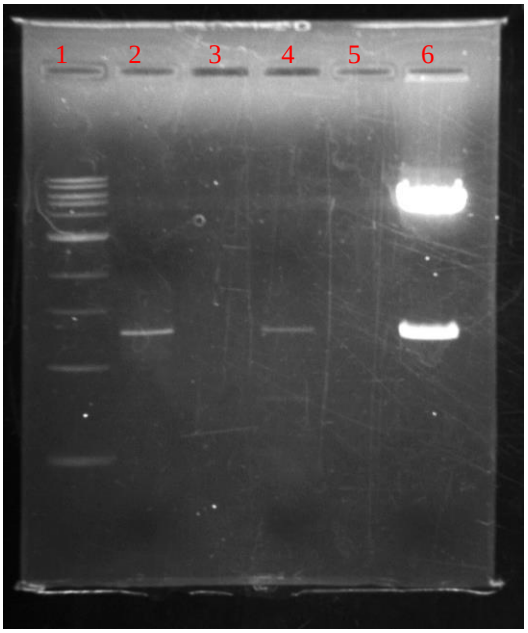


Figure 5. Gel image of the successful ligations of sew PCR products for two candidate mutants. Lane 1 contains the 100 bp ladder. Lanes 2 and 3 are two successful ligations of KHF to WYY products. Lanes 3 and 4 are two successful ligations of D to M products.

3.2 Candidate Mutant Analysis

3.2.1 ligation of candidate mutant cDNA of Gα12 into pcDNA 3.1 plasmid

Restriction digests performed on KHF to WYY and D to M candidates resulted in fragments of predicted length. Figure 6A depicts successful restriction digests of KHF to WYY, D to M and pcDNA 3.1 plasmid with the enzymes NotI and KpnI. Restriction digest of candidate D to M was performed a second time after no colonies were observed following bacterial transformation (Figure 6B). Bands found at approximately 1.2 kb were cut from the gel as well as the large band found at 5.5 kb. These fragments were used in the ligation reactions after gel extraction. Results of the ligation reactions were not pictured, but were found to be successful for both mutant candidates after viewing in the Kodak gel documentation system.

A. 

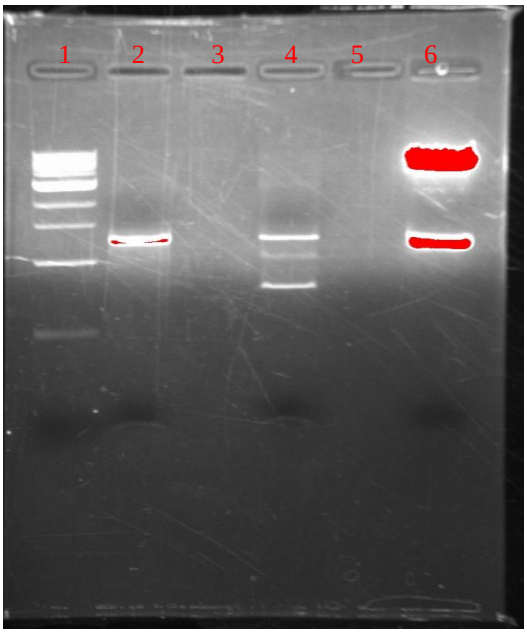
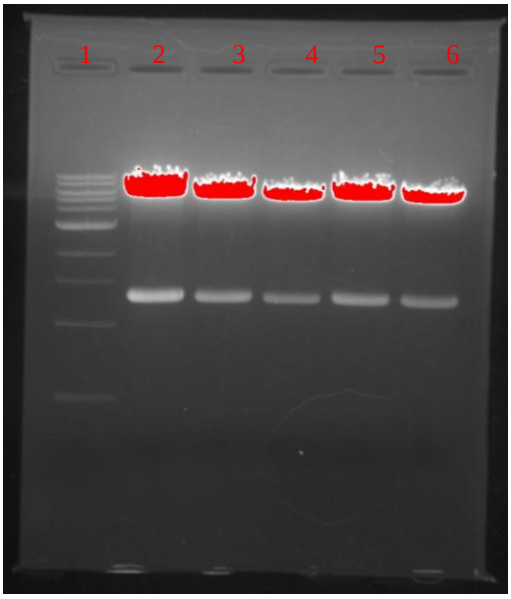
B. 

Figure 6. Results of restriction digest reactions on candidate mutants and pcDNA 3.1. A depicts restriction digest of KHF to WYY in lane 2, D to M in lane 4 and plasmid pcDNA 3.1 in lane 6 with enzymes NotI and KpnI. B depicts a second restriction digest of D to M in lane 2 and plasmid pcDNA 3.1 in lane 6 with enzymes EcoRI and KpnI. Lane 1 in both gels contains the 1 kb ladder.

3.2.2 diagnostic restriction digests

Restriction digests were performed on plasmids isolated from colonies produced following bacterial transformation. Plasmids that were cut correctly by the restriction enzymes were sent for sequencing. Figure 7 displays the results of these restriction digests. A depicts the successful digest of KHF to WYY in lanes 2-6. Restriction enzymes used in Figure 7A were NotI and KpnI. B depicts the digest of D to M in lanes 7-9**.** Restriction enzymes used in Figure 7B were EcoRI and KpnI.

Lane 2 (Figure 7A) contained the candidate mutant insert chosen to be sequenced for the KHF to WYY candidate. Analysis of the primary sequence of the selected insert by pairwise alignment to native Gα12 primary sequence revealed the insert successfully contained the desired Gα12-to-Cta mutation (Figure 8). Lane 7 (Figure 7B) contained the candidate mutant insert chosen to be sequenced for the D to M candidate. Analysis of the primary sequence of the selected insert by pairwise alignment to native Gα12 primary sequence revealed the insert successfully contained the desired Gα12-to-Cta mutation (Figure 8).

A. 

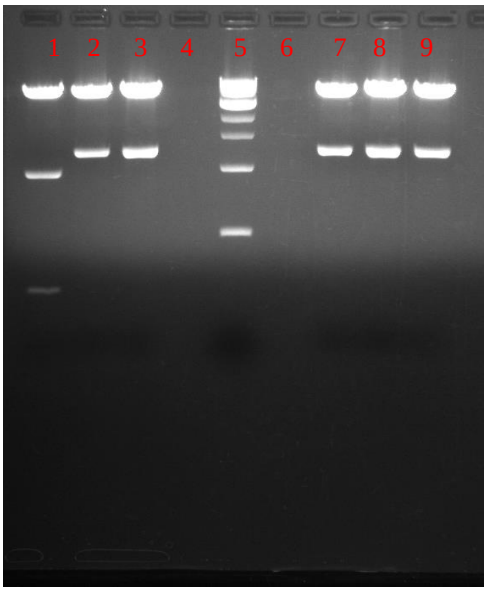
B. 

Figure 7. Gel images of diagnostic restriction digests of plasmids elucidated from selected colonies produced following bacterial transformation. A depicts that all KHF to WYY candidate mutants were successfully digested with enzymes NotI HF and KpnI HF. B depicts successfully digested D to M mutant candidates with EcoR1 and KpnI in lanes 7-9. A 1 kb ladder was used for both gels.

K^{310}HF to WYY

```
                70        80        90       100       110       120
KHF     LFFNVSIILFLNKMDLLVEKVKSVSIKWYYPDFKGDPHRLEDVQRYLVQCFDRKRRNRSK
        :::::::::::::::::::::::::::: ..:::::::::::::::::::::::::::::
Native  LFFNVSIILFLNKMDLLVEKVKSVSIKKHFPDFKGDPHRLEDVQRYLVQCFDRKRRNRSK
              290       300       310       320       330       340
```

D^{334} to M

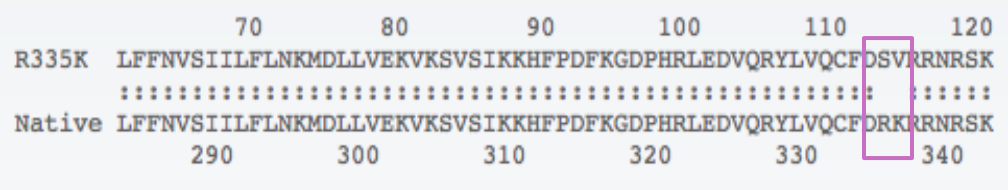
```
              290       300       310       320       330       340
Ga12    LFFNVSIILFLNKMDLLVEKVKSVSIKKHFPDFKGDPHRLEDVQRYLVQCFDRKRRNRSK
        :::::::::::::::::::::::::::::::::::::::::::::::: :::: ::::::::
DtoM    LFFNVSIILFLNKMDLLVEKVKSVSIKKHFPDFKGDPHRLEDVQRCLVQCFMRKRRNRSK
                70        80        90       100       110       120
```

Figure 8. Alignment of candidate mutant Gα12 primary sequences to native Gα12 primary sequence for KHF to WYY and D to M. The mutation of interest is shown outlined in purple.

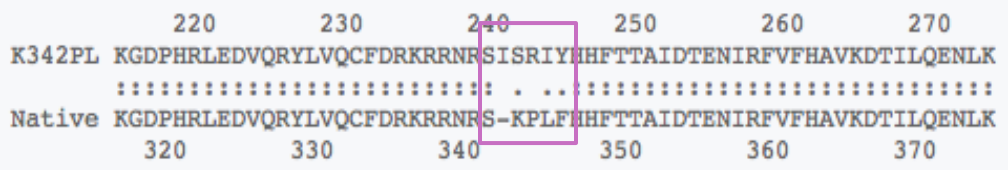
3.3 Analysis of Candidate Mutants from Fall 2013 BIOL344

All of the candidate mutants sent for sequencing from the Fall 2013 BIOL344 class were deemed successful after alignment to native Gα12 primary sequence (Figure 9).

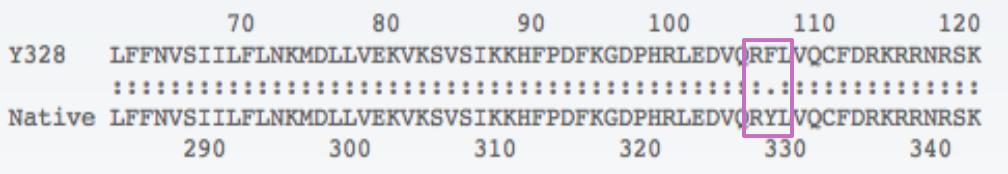
$R^{335}K$ to SV



$K^{342}PLF$ to ISRIY



Y^{328} to F



$N^{339}R$ to SS

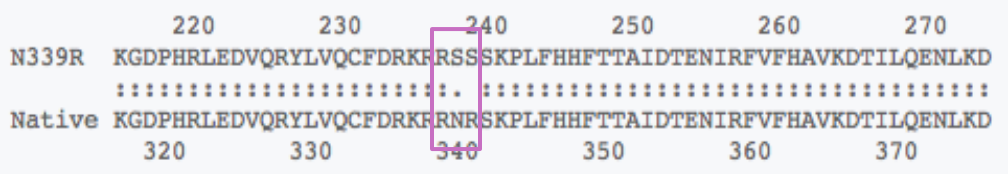


Figure 9. Alignment of candidate mutant Gα12 primary sequences to native Gα12 primary sequence. The mutation of interest is shown outlined in purple.

4. Discussion

From the experimental techniques deployed in this study, it can be deduced that oligonucleotide mutagenesis is sufficient in constructing Gα12-to-Concertina mutants. Furthermore, it was found that if oligonucleotide mutagenesis is unsuccessful, sew PCR, a more complicated strategy, can be used to successfully create Concertina substitutions within the C-terminal 42-amino acid region of Gα12. Future work must be performed to create a new D^{334} to M mutation without the unwanted mutation that resulted in previous sewing PCR attempts. This mutation error may have been caused by either an error that occurred during the PCR amplification, or the designed oligonucleotides contained a sequencing mistake.

Upon successful mutagenesis of each particular amino acid sequence, future experiments may be executed to determine the necessity and sufficiency of the sequence in the activity of Gα12. Such experiments could incorporate methods like those of Jones and Gutkind, who altered amino acid sequences in the GTP-binding domain ($Q_{229}L$) of Gα12. Their team transfected mutated Gα12 cDNA into tissue cells and then employed metabolic labeling and cell fractionation to then determine activity within the cell[6]. Further experiments may help to determine whether specific amino acid sequences are necessary or sufficient for Gα12 action, as well as determine if other factors are at work in the accompanying mechanisms of cell mutation and proliferation. As seen in work by Lopez *et al.*, particular amino

acid sequences may serve to elucidate the mechanisms of Gα12 and its relationship with various cell functions; in this case, the activation of the Ras protein[7]. If similar experiments continue to be performed utilizing many different amino acids within Gα12, we may begin to understand the specific molecular processes associated with G proteins, specific amino acids within them, and subsequent cell proliferation signaling as instigated by the serum response factor. Further understanding of the C-terminal region of Gα12 may result in targets for future antineoplastic drugs.

5. Acknowledgements

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