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Characterizing Gα12 and p114RhoGEF's Specific Interaction Through Site-Directed Mutagenesis

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Abstract

G proteins are heterotrimeric proteins that signal for a variety of important processes in the cell. There are four subfamilies of G proteins in mammals, including the G12/13 subfamily that consists of Gα12 and Gα13 and has been implicated in cancerous progression. There are many proteins that interact with Gα12, among these the closely related Rho-specific guanine nucleotide exchange factors p114RhoGEF and AKAP-Lbc. Although the structural features of the interaction between Gα12 and p114RhoGEF have yet to be determined, we have found that p114RhoGEF and AKAP-Lbc utilize a region closely homologous between these proteins for interaction with Gα12. Using a PCR-based strategy, we engineered point mutations to create charge-substitutions of amino acids identical in AKAP-Lbc and P114RhoGEF. These constructs were then utilized for protein interaction experiments to determine which constructs showed decreased binding affinity for Gα12. To examine the specificity of Gα12 interaction for these RhoGEF proteins, we performed G protein driven assays of serum response factor mediated transcriptional activation. These results showed that a dominant-negative p114RhoGEF preferentially blocked the Gα12-mediated stimulation of serum response factor in comparison to Gα13, suggesting that specific binding to p114RhoGEF plays a role in the Gα12-specific mechanism of this signaling pathway. The long-term purpose of this research is to characterize the structural interaction between Ga12 and P114RhoGEF and use this knowledge to further investigate the role of Ga12-p114RhoGEF binding in different cell types.

Keywords: G-proteins, p114RhoGEF, site-directed mutagenesis

1. Introduction

G proteins are heterotrimeric cell signaling proteins that are comprised of three subunits, α, β and γ.[1] The G proteins are bound to a transmembrane protein called a G protein-coupled receptor (GCPR) that is comprised of 7 transmembrane α-helices. GPCRs are stimulated outside the cell through hormones or other ligands and once stimulated, the G protein then drives specific cellular processes as seen in Figure 1. Due to G proteins' nature, being essential signaling proteins, they are involved in many physiological processes such as controlling muscle contraction, vascular dilation, heart rate regulation, sensory inputs, and cell growth among other functions.[2]

Gα12 is a member of the G12/13 subfamily of G proteins that interacts with many intracellular target proteins.[3] Overexpression or activating mutation of Gα12 leads to oncogenic transformation. One of the proteins reported to bind Gα12 is AKAP-Lbc, a protein strongly correlated with the disease cardiac hypertrophy. This disease causes a change in cardiac myocyte behavior; causing secretion of excess extracellular matrix which in turn stiffens the heart tissue and contributes to heart failure.[4] Our lab has identified a binding domain in AKAP-Lbc specific to Gα12. By performing a BLAST analysis on axin, a protein that binds to Gα12, it was found that a small region in the carboxy-terminus of the protein had slight homology with AKAP-Lbc. With this information a BLAST analysis was done on this amino acid sequence to identify other proteins with close homology to this specific Gα12-binding region. BLAST

is a program that identifies other proteins with similar homology on the amino acid sequence entered and lists the names of the proteins and area of homology on the found protein. P114RhoGEF was found to contain very close homology to AKAP-Lbc's binding domain that spans 106 amino acids long. Further protein-protein interaction experiments demonstrated that this P114RhoGEF domain along with AKAP-Lbc did in fact bind to Gα12.
The purpose of this research is to characterize the interactions between Gα12 and p114RhoGEF and show the similarities in binding Gα12 with p114RhoGEF and AKAP-Lbc through a novel, non-regulator of G protein signaling (RGS) domain. Determining the amino acids that contribute to the binding of p114RhoGEF to Gα12 could provide further understanding of the physiological and pathological roles in different cells. In order to identify which amino acids on p114RhoGEF are binding to Gα12, site targeted mutagenesis was performed on the binding region proposed by previous lab members. Three different mutants were engineered to through the use of polymerase chain reaction-splicing by overlap extension (PCR-SOEing). Any changes in binding affinities between mutated p114RhoGEF and Gα12 will be quantified through the use of pull-down experiments.

2. Materials and Methods

2.1 PCR-SOEing

The mutants were engineered through the use of PCR-SOEing. The primers needed for PCR were created at the site of the mutant. Primers contained the proper mutations that allowed for the mutation of only one mutant per reaction. A forward and a reverse primer for every mutant was created. The 3 mutants needed gave 6 total reactions, 3 forward reactions and 3 reverse reactions. Every reaction contained 5 µL of 10x PFU buffer, 1.5 µL of 10mM dNTP mix, 1µL of 25 ng/µL p114RhoGEF parental DNA, 2.5 µL of DMSO, .79 µL of PFU Turbo Cx (DNA polymerase), 5µL of 5mM of each forward and reverse oligonucleotide, and 29.2 µL of DI water to make a total of 50 µL per reaction. The PCR machine, Bio-Rad T100™ Thermal Cycler, was set to a melting temperature of 94°C for 45 seconds, an annealing temperature of 53°C for 1 minute, an elongation temperature of 72°C for 1 minute, these steps were repeated 27 more times, and after the 27 repeats a final elongation of 3 minutes. The DNA was then kept refrigerated at 4°C until transferred to a -20°C freezer. A 1.2% agarose gel-electrophoresis was used for verification of proper DNA PCR product base-pair lengths. A 100 base-pair (bp) ladder was used to measure band sizes. A 1:5 ratio of Blue-Orange dye to DNA product was used per gel load sample, so 5 µL of dye: 25 µL of PCR product. The DNA was then cut out from the gel and purified for the use of the SOEing section of the mutagenesis. The DNA was obtained through the use of Promega Wizard SV columns to bind the DNA and then eluted from the column through the use of 50 µL of Qiagen Elution Buffer (EB). The final step to PCR-SOEing was to combine each half of the mutant p114RhoGEF in order to obtain the whole p114RhoGEF DNA sequence. Same parameters were held as the previous PCR cycle. The 2 differences in the SOEing PCR cycle was the elongation was changed from 1 minute to 2 minutes due to the longer DNA chain and the final elongation was changed from 3 minutes to 6 minutes for the same reason. The final DNA product was obtained through gel-electrophoresis and purified using the same previous methods.

2.2 Ligation of p114RhoGEF into PGEX-KG plasmid

The p114RhoGEF mutants were spliced into a plasmid, PGEX-KG. Restriction enzymes HindIII and EcoRI were used to digest the mutants to rid of excess nucleotides not pertaining to the p114RhoGEF coding DNA. Each mutant digestion had a total volume of 30 µL consisting of 3 µL of NEBuffer 2.1, .7 µL of HindIII, .7 µL of EcoRI-HF, and 25.6 µL of PCR sample mutant. Each digestion was placed in a 37°C water bath for 2 hours. The DNA needed was extrapolated using gel-electrophoresis and a 1kbp ladder to determine proper size. The proper gel band was cut out for each mutant and then purified using same methods as mentioned in PCR-SOEing. PGEX-KG was also digested using HindIII and EcoRI restriction enzymes. The reaction was 30 µL in total volume containing 3 µg of plasmid DNA, 3 µL of NEBuffer 2.1, .7 µL of EcoRI, .7 µL of HindIII, and the rest of the volume was made up using DI water. The digestion was placed in a 37°C water bath, after 1 hour 1.5 µL of CIP was added and then placed back in the water bath for another hour. The final plasmid DNA was recovered using the same technique as the mutant DNA mentioned previously. The ligations were then performed. 5 µL of mutant DNA was placed in a microfuge tube with 2µL of 10x Ligase Buffer, .7 µL of DNA ligase, 3.68 µL of the plasmid, and 8.62 µL of DI water. A control of only plasmid with no insert, no mutant DNA, was used, the lack of 5µL of mutant DNA was replaced by 5 µL of DI water. The reaction microfuge tubes were left at room temperature for 1 hour for maximum enzyme activity and then placed in the fridge

overnight for maximum enzyme activity. The final product was obtained using the same gel-extraction and purification methods mentioned previously.

2.3 Transformation of ligations into JM-109 *E. Coli*

Obtained one cold LB-Ampicillin plate per mutant DNA sample and set out to allow to warm up to room temperature. A single aliquot of 100 µL JM-109 cells was obtained and distributed evenly in three smaller aliquots. Cells were placed in 14mL Round-Bottom Falcon Tubes and kept in ice as much as possible. 1 µL of each final ligation product was dispensed unto the cells, making sure not to cross-contaminate the different ligation products. The cells were then kept on ice for 30 minutes. The cells were then heat-shocked. Falcon tubes were placed in 42°C bath for 45 sec then placed immediately back into ice for 2 minutes. 0.7 mL of SOC medium were pipetted into each Falcon tube and incubated at 37°C using a Brunswick Innova 4000 shaking incubator at 230 rpm, for 1 h. Each aliquot was then placed into a microfuge tube and spun at 9000g for 3 minutes. All the supernatant was discarded besides 100 µL. The cells were re-suspended and pipetted unto each LB-Ampicillin plate and then placed in the 37°C incubator for 14 hours.

2.4 Obtaining each mutant plasmid from JM-109 *E. Coli*

QIAprep Spin Miniprep Kit was used for the entire procedure. Qiagen P1, P2, N3, PB, PE, and EB buffers were used to lyse the cells obtained from a 2 mL culture. P1 buffer suspends the cells in solution, P2 is the basic lysis buffer, N3 is an acidic buffer used to neutralize the lysis buffer, PB buffer causes binding of the plasmid DNA to the columns of the Miniprep Kit, the PE buffer washes the column, and the EB buffer elutes the DNA off of the columns into a microfuge tube. 250 µL of P1, 250 µL of P2, 350 µL of N3, 500 µL of PB, 750 µL of PE, and 50 µL of EB buffer were used. DNA was stored in a -20°C freezer.

2.5 DNA Sequencing and verification

DNA plasmids were sequenced through GeneWiz, South Plainfield, NJ. The DNA results were translated using EMBOSS Transeq and aligned, with respect to the native p114-RhoGEF amino acid sequence, using ExPASy LALIGN to verify no other mutations were present.

2.6 Transformation of BL-21 Gold Cells

An aliquot of BL21 Gold DE3 cells was obtained, thawed out, and dispensed equally into 14 mL Falcon tubes that had been chilling in ice. 1 µL of each mutant glutathione-S-transferase (GST)-fusion DNA plasmid at a concentration of 10 ng/µL was dispensed in its perspective Falcon tube. Each Falcon tube was then flicked to mix the plasmid DNA with the BL-21 Gold cells and placed back in ice for 30 minutes. The cells were then heat-shocked for 20 seconds in a water bath set at 42°C and then placed immediately in ice for 2 minutes to incubate. 0.8 mL of SOC medium was dispensed into each Falcon tube. All tubes were then placed in a shaker incubator, New Brunswick Scientific I2400 Incubator Shaker, set at 230 rpm and 37°C for approximately 1 hour. 50 µL of the recovered cells from each Falcon tube was dispensed onto their respective LB-Ampicillin agar plates. The solution was dispersed throughout using sterilized glass beads and then left to sit for approximately 5 minutes. The plates were then placed in the incubator for 12-14 hours.

2.7 Immobilization of GST-p114RhoGEF mutant proteins

All glassware, plastic containers, and pipet tips were sterilized through the use of an autoclave. From the BL-21 Gold DE3 cell transformations 1 colony was picked from each mutant construct and placed in a 14mL Falcon tube containing 2 mL of LB media and ampicillin at a concentration of 75 µg/mL. The Falcon tubes were placed in the shaking incubator, New Brunswick Scientific I2400 Incubator Shaker, set at 37°C and 230 rpm for approximately 14 hours. 1.5 mL of each culture was then transferred to a 500 mL Erlenmeyer flask containing 120 mL of LB and placed back in the shaker for 90 minutes. Using a spectrophotomer, Spectronic Genesys 2, the absorbance at 600 nm was taken until the absorbance of each culture was between 0.5-0.8, LB media was used to zero the instrument. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.5 mM to allow for expression of our GST-fusion plasmids. The cultures were then left to incubate for 3 hours. For the remaining of the steps the bacterial cultures were kept in ice at all times. The cultures were centrifuged and lysed using 4-5 mg of lysozyme and placed

back in ice for 1 hour. The cultures were then centrifuged and decanted of their supernatant into a 15 mL conical tubes containing 0.15 mL of glutathione-sepharose beads. The conical tubes were placed in the Orbitron for 45 minutes to allow the GST fused p114RhoGEF proteins to bind to the glutathione-sepharose beads. The beads were then washed with TED buffer and then washed with TED buffer containing NaCl at a concentration of 150 mM to remove any interactions between non-specific binding of proteins. The beads were then dispensed in 50 µL aliquots and snap-frozen in liquid nitrogen and stored at -80°C.

2.8 p114RhoGEF and Gα12 Protein Interaction Assays

GST-p114RhoGEF mutant proteins were suspended in HEDM along with Gα12 and a blank HEK293 cell lysate, not containing Gα12. These proteins were placed in the Orbitron to rock for 2 hours to allow for any interaction, if any, to occur. The interaction microfuge tubes were then centrifuged and washed using HEDLM. 1M of dithiothreitol (DTT) and a 4x Sample buffer was added to each interaction tube and placed in a 72°C water bath to incubate. Each sample was then placed in a 12% polyacrylamide gel to allow for protein separation, for approximately 90 minutes, or until complete, a 1x SDS-PAGE buffer was used. Following the protein separation a western blot, or immunoblot, analysis was performed. The 1° antibody was Santa Cruz sc-409, a Gα12 antibody derived from rabbit. A 2° antibody, anti-rabbit AP-conjugated antibody, was used. Lastly to develop the immunoblot a 2:1 ratio of NBT:BCIP was used and placed on the shaker for approximately 10 minutes. The results were captured through Kodak Gel Logic 100 Imaging System.

3. Results and Discussion

Through the use of PCR-SOEing the mutants were engineered (Figure 1). Each band was the appropriate

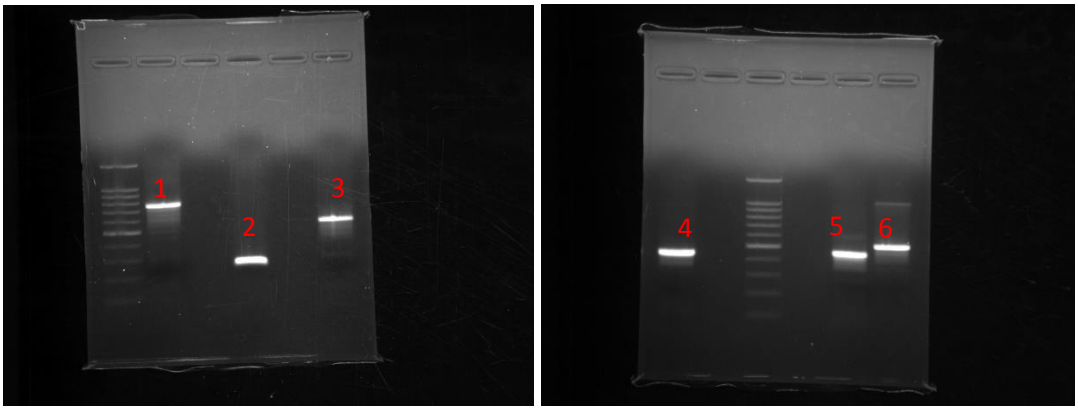

Figure 1: Gel electrophoresis of forward and reverse reaction PCR for each mutant. Number 1 and 2 are for E695R, 3 and 4 for E759R, and 5 and 6 pertain to E789K.

size corresponding to the positioning of mutant in the DNA sequence of p114RhoGEF. The 2 bands that pertain to each mutant, as mention in Figure 2, add up to just below 1000bp which is what we are needing since the addition of the DNA of p114RhoGEF, 777bp, and the DNA of the plasmid, 200bp, gives 977 base pairs total. The brighter bands are the correct DNA size so they were carved out of the gel and purified as mentioned in the **Methods** section. The

DNA was used for the SOEing portion of the experiment and yielded positive results as seen in Figure 2.

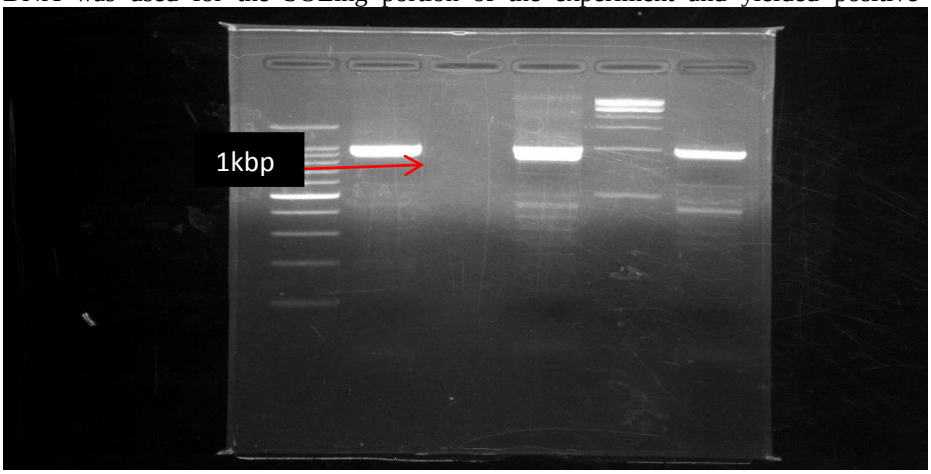

Figure 2: DNA bands after the second PCR. As expected the bands needed are just under the 1kbp ladder band as pointed by the red arrow.

The gel in Figure 2 shows the final SOEing product which yields a band right under the 1000 base pair band on the ladder which confirms that we have the proper amount of base pairs. The plasmid PGEX-KG, along with the PCR products were digested with 2 enzymes, EcoRI and HindIII(Figure 3). These 2 enzymes cut on two sites of the PGEX-KG plasmid which gives an open plasmid, and it gets rid of the excess DNA on the PCR-SOEing products since it contained 200bp of PGEX-KG to give only the DNA of p114RhoGEF.

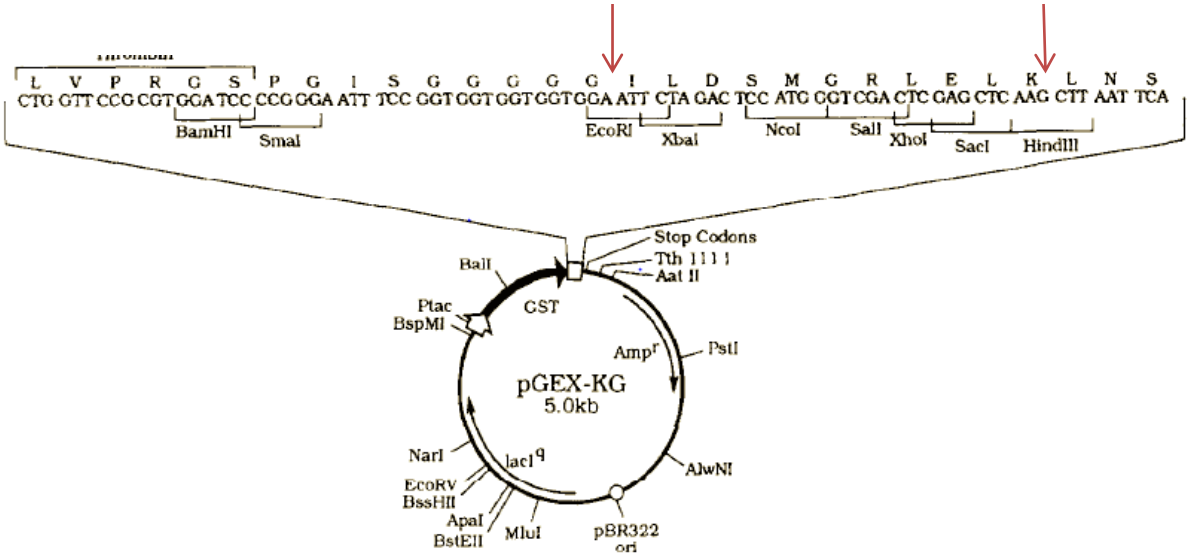

Figure 3: Red arrows show where on the circular plasmid the restriction enzymes cut and in between these two sites is where the DNA for p114RhoGEF was ligated.[5]

After the digestions, p114RhoGEF DNA was ligated into the plasmid and then to verify the ligations were a success, a gel was run containing the ligation products. Figure 4 shows the products and their sizes.

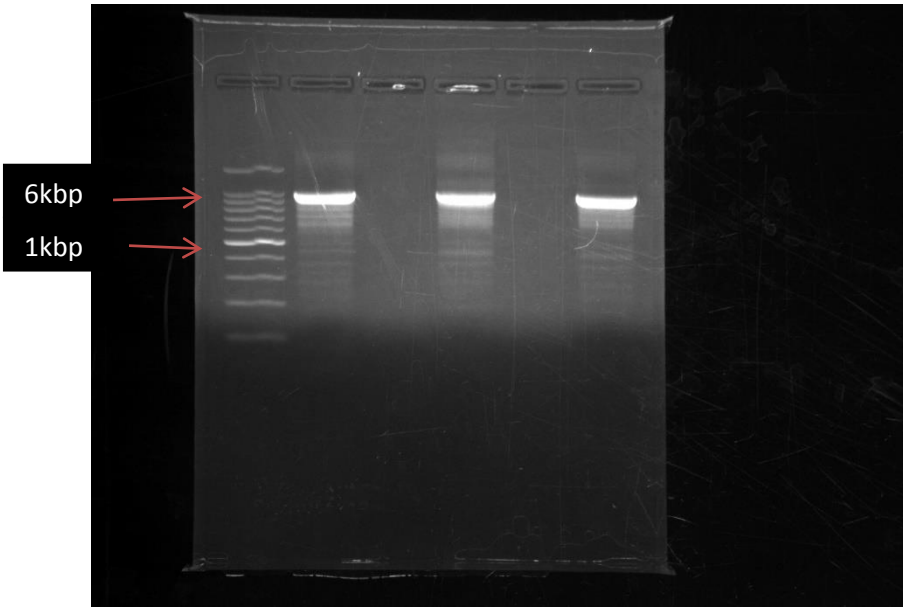

Figure 4: The bright bands show each mutant DNA and the size of each. They are all the same length and are below the 6000bp band.

The bright bands again show the most abundant product after the ligations. All three ligations were successful. The bright bands are located in between the 5kbp and the 6kbp bands on the ladder meaning the product size is in between 5000 and 6000 base pairs. This is a positive result because we know the size of the plasmid is 5000bp and the DNA is 777bp meaning that the total length of the plasmid should be just under 6kbp which is confirmed by the fluorescent bands on the gel. After confirming that the size of our plasmid is correct the 3 mutants were sent to an outside lab (GeneWiz, South Plainfield, NJ) to sequence the p114RhoGEF DNA that was spliced into the PGEX-KG plasmid. The DNA sequence received showed only nucleotide changes in the area where the mutation was engineered the rest of the DNA matched up identically to the parental p114RhoGEF DNA meaning that the SOEing PCR was successful (Figure 5).

p114RhoGEF (Thr-686 to Leu-791; 106 amino acids)

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T   R   G   N   L   L   L   E   Q   E   R   Q   R   N   F   E   K   Q   R   E
acg cgt ggg aac ctg ctg ctg gag cag gag cgg caa cgc aac ttc gag aag cag cgg gag

E   R   A   A   L   E   K   L   Q   S   Q   L   R   H   E   Q   Q   R   W   E
gag cgc gcg gcc ctg gag aag ctg cag agc cag ctg cgg cac gag cag cag cgc tgg gag

R   E   R   Q   W   Q   H   Q   E   L   E   R   A   G   A   R   L   Q   E   R
cgc gag cgc cag tgg cag cac cag gag ctg gag cgt gcg ggc gcg cgg ctg cag gag cgc

E   G   E   A   R   Q   L   R   E   R   L   E   Q   E   R   A   E   L   E   R
gag ggc gag gcg cgg cag cta cgc gag cgg ctg gag cag gag cgg gcc gag ctg gag cgc

Q   R   Q   A   Y   Q   H   D   L   E   R   L   R   E   A   Q   R   A   V   E
cag cgc cag gcc tac cag cac gac ctg gag cgg ctg cgc gag gcc cag cgt gcc gtg gag

R   E   R   E   R   L
cgc gag cgg gag cgc ctg
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Figure 6: p114RhoGEF 106 amino acid region that interacts with Gα12 and homologous to AKAP-Lbc. Highlighted in yellow are all the mutants we currently engineered.

Each mutant was sequenced and verified using the same methods as previously mentioned. Figure 6 shows every mutant of p114RhoGEF that we managed to engineer. Each of these mutants are homologous in both proteins, AKAP-Lbc and p114RhoGEF, when their amino acid sequences are aligned.

After the expression of the p114RhoGEF mutated plasmid constructs we were able to perform protein interaction assays to determine whether the change in amino acid would lead to a lower binding affinity to Gα12. The results are shown in Figure 7.

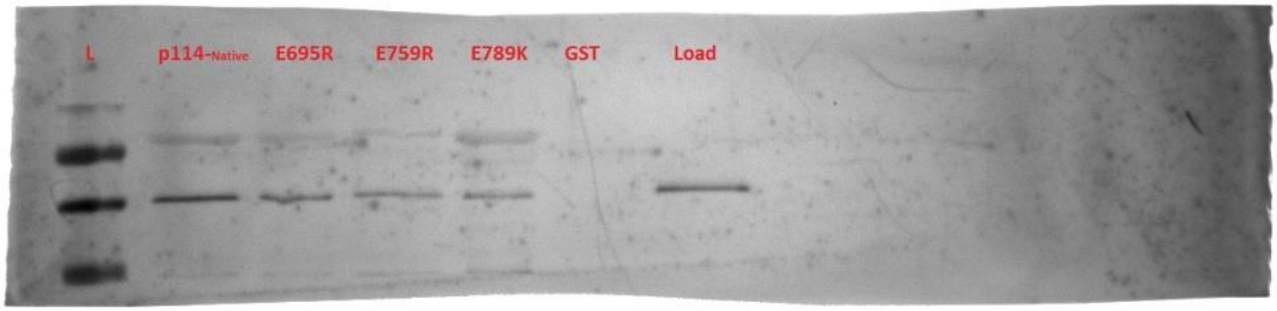

Figure 7: The results from a pulldown of $G\alpha12^{QL}$ using p114RhoGEF native protein and its 3 mutants, E695R, E759R, E789K, and GST as a negative control.

The data shown in **Figure 7** shows possible loss of binding affinity to $G\alpha12^{QL}$ with mutants E759R and E789K and could be of use in later comparisons as we continue to change amino acids in p114RhoGEF that are homologous to AKAP-Lbc. Complete loss of binding to $G\alpha12^{QL}$ does not occur and thus eliminates the possibility that one of these mutants is essential to the binding of $G\alpha12^{QL}$. However, it could be that one of these mutants helps stabilize the conformational changes experienced during the interaction of these two proteins and results in p114RhoGEF having slightly less binding affinity.

Including the three mutants mentioned earlier, E695R, E759R, and E789K, four other mutants were also engineered using the same methods. The amino acids changed were R698E, E701R, E746R, R760E. R760E was specially chosen due to research showing Rgnef, a protein with close homology to AKAP-Lbc, losing binding to Gα13 when this mutation occurs. This residue is conserved in Rgnef, AKAP-Lbc, and p114RhoGEF.[6] Though previous research in our lab shows preferential binding of AKAP-Lbc to Gα12 than to Gα13, we proceeded to engineer both constructs, p114RhoGEF R760E, and AKAP-Lbc R2641E. The results of the pulldown experiment with $G\alpha12^{QL}$ are shown in Figure 8.

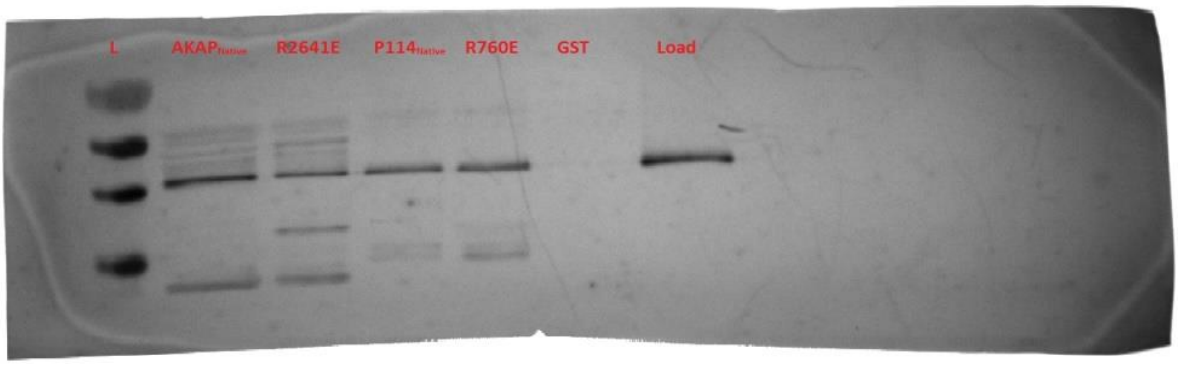

Figure 8: Pulldown of $G\alpha12^{QL}$ with AKAP-Lbc^{257}, AKAP-Lbc R2641E, p114RhoGEF, p114RhoGEF R760E, and a negative control, GST.

The results acquired from the immunoblot, Figure 8, provides further affirmation of our claims that AKAP-Lbc and p114RhoGEF preferentially bind to Gα12 through a different mechanism as there was no change in binding affinities to $G\alpha12^{QL}$ in either mutant, AKAP-Lbc or p114RhoGEF. The similar binding affinities in AKAP-Lbc and p114RhoGEF shown in the immunoblot provides certainty that these two proteins do behave very similarly in the 106 amino acid region of close homology. A pulldown experiment using Gα12 and Gα13 in one immunoblot and using the same antibody for both G proteins would provide the evidence needed to distinguish the differences in binding affinities of our two proteins of interest p114RhoGEF and AKAP-Lbc.

4. Conclusion

These results show two definitive residues, E759R and E789K, that diminish the binding of p114RhoGEF to $G\alpha12^{QL}$. These residues seem to disrupt binding to $G\alpha12^{QL}$ and are needed for $G\alpha12^{QL}$ to properly bind p114RhoGEF. However, complete loss of binding to $G\alpha12^{QL}$ does not occur and thus eliminates the possibility that one of these mutants blocks the ability for $G\alpha12^{QL}$ to bind p114RhoGEF or that the residue changes the conformation of the binding region.
Further protein assays need to be performed with the remaining three p114RhoGEF mutant constructs, R698E, E701R, and E746R. These three constructs pertain to three AKAP-Lbc mutants that showed decreased binding affinity to $G\alpha12^{QL}$ in pulldown experiments performed by Autumn Towne, whose focus is on AKAP-Lbc and its mutants. Similar results should lead to a definitive conclusion in that AKAP-Lbc and p114RhoGEF bind $G\alpha12^{QL}$ through the same mechanism. Similar behavior has been shown in our lab that mutations in $G\alpha12^{QL}$, C11A, causes both AKAP-Lbc and p114RhoGEF to lose its strong binding affinity to $G\alpha12^{QL}$. This similar behavior strengthens our hypothesis that these two proteins use the same binding mechanism. In the future we will create a mutant construct of p114RhoGEF containing the 2 mutants that showed decrease of binding to $G\alpha12^{QL}$ and see whether this blocks the binding to $G\alpha12^{QL}$ completely.

5. Acknowledgements

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