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Determinants of Gα12 specific interaction in the RhoGEF AKAP-Lbc

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Abstract

AKAP-Lbc is a Rho-specific guanine nucleotide exchange factor (RhoGEF) that is stimulated by trimeric G protein alpha subunits of the G12/13 subfamily. Despite lacking a RGS-homology domain that is well characterized as allowing several other RhoGEFs to interact with G12/13 alpha subunits, AKAP-Lbc has been reported as a specific binding partner of G alpha 12. This protein interaction appears to play a role in cardiac hypertrophy as well as heart development. In cardiac hypertrophy, damage to the cardiomyocytes initiates a signaling cascade that results in these cells becoming more fibroblast-like and secreting extracellular matrix proteins, leading to the stiffening of heart tissue. We have identified a G alpha 12 binding region within AKAP-Lbc that shares homology with p114RhoGEF, a protein that also lacks a RGS-homology domain. Based on our initial findings that p114RhoGEF also binds G alpha 12, we aligned these proteins to identify amino acids within the G alpha 12 binding region of AKAP-Lbc that are shared with p114RhoGEF and engineered charge-substitution mutants at these positions. Our examination of these mutants in protein binding assays has revealed several amino acids in AKAP-Lbc that are important in G alpha 12 interaction. Our goal is to use this information to better characterize the structural features of the interaction between G alpha 12 and RhoGEFs which lack RGS-homology domains and ultimately to manipulate this protein interaction to decipher its role in cellular signal transduction.

Keywords: Gα12, AKAP-Lbc, RhoGEF

1. Introduction

Heterotrimeric guanine nucleotide-binding (G) proteins are integral membrane proteins that play a major role in activating and defining cellular response to extracellular stimuli. Upon activation of a G-protein-coupled receptor (GPCR), one of the three G protein subunits, α, exchanges GDP for GTP and dissociates from the other two subunits, β and γ.[1] Once the α subunit dissociates, it then serves as a signaling molecule for downstream effectors. Gα12 is a member of the G12/13 class of G proteins and has been shown to play roles in cell growth, apoptosis, oncogenic transformation, and stress fiber formation.[2]

A-kinase-anchoring-proteins (AKAPS) are a group of functionally related proteins that coordinate cAMP-responsive events at defined subcellular compartments by directing protein kinase A to its preferred substrate.[3] In particular, AKAP-Lbc is a splice variant of the Lbc family of Rho-specific guanine nucleotide exchange factors (RhoGEF), allowing it to act as both a protein kinase A anchoring protein and a RhoGEF. The AKAP-Lbc complex is activated in response to a signal molecule binding to the extracellular portion of a GPCR, which releases Gα12 from the membrane and binds with AKAP-Lbc. AKAP-Lbc then facilitates Rho to exchange GDP for GTP and the activated Rho may then participate in multiple cellular processes. This signaling pathway is of particular importance because it has been shown to play a role in initiating cardiac hypertrophy.[4] In cardiac hypertrophy, damage to the cardiomyocytes initiates a signaling cascade that results in a pathological remodeling process that involves the re-expression of a fetal

gene program that cause the cells to become more fibroblast-like and to secrete proteins into the extracellular matrix. [4] This leads to the stiffening of heart tissue that can eventually culminate in cardiac dysfunction and heart failure. [4]

What separates AKAP-Lbc from most Gα12-binding RhoGEFs is its absence of an RGS homology (RH) domain.[3] While the interaction between Gα12 and RhoGEFs containing RH domains, such as LARG and p115, has been well characterized, how Gα12 interacts with proteins missing this domain is still not well understood.[5] p114RhoGEF, which also lacks an RH domain, has been found by previous members of this lab to bind Gα12 as well. When the amino acid sequences of p114RhoGEF and AKAP-Lbc were compared, there was found to be a 106 amino acid region of close homology (47% identity) between the two proteins. To determine if this region of homology was important to the protein's ability to bind Gα12, charge substitutions were made at eleven residues present in both AKAP-Lbc and P114RhoGEF. Co-precipitation assays were then used to measure the ability of the AKAP-Lbc variants to bind Gα12.

2. Materials and Methods

2.1 Creation of GST AKAP Fusion

Eleven charge substitutions were made in the 106 amino acid region of homology in AKAP-Lbc. GST-fusions were created by polymerase chain reaction (PCR)-amplifying a 771bp region encompassing the c-terminal 257 amino acids of AKAP that included the region of homology shared with p114. On either side of the 771bp region, EcoRI (5'-GAATTC-3') restriction endonuclease sites were engineered to allow ligation into pGEX-kg plasmid vector (GE Healthcare). Oligonucleotides were selected using the published AKAP sequence to hybridize to the nucleotide sequence. The PCR oligonucleotides used (Eurofins Genomics) are as follows:

E2574R: 5'-CCG CCC GAG CTC CCT GAT CAG GCA GGA GAA GCA GCG C-3'
3'-GGC GGG CTC GAG GGA CTA GTC CGT CCT CTT CGT CGC G-5'
E2629R: 5'-TGG CCC AGC GCG AGG AAC GCG TGC AGC AGG GGC AG-3'
3'-ACC GGG TCG CGC TCC TTG CGC ACG TCG TCC CCG TC-5'
E2582R: 5'-GAA GCA GCG CAG CCT TCG AAA GCA GCG CCA GGA C-3'
3'-CTT CGT CGC GTC GGA AGC TTT CGT CGC GGT CCT G-5'
R2579E: 5'-GAG CAG GAG AAG CAG GAA AGC TTG GAG AAG CAG CGC CAG-3'
3'-CTC GTC CTC TTC GTC CTT TCG AAC CTC TTC GTC GCG GTC-5'
E2640R: 5'-GCA GGA CCT GGA AAA GCG TCG CGA GGA GCT CCA GCA G-3'
3'-CGT CCT GGA CCT TTT CGC AGC GCT CCT CGA GGT CGT C-5'
E2627R: 5'-CCT CCT GGC CCA GCG TCG CGA GGA GGT GCA GCA G-3'
3'-GGA GGA CCG GGT CGC AGC GCT CCT CCA CGT CGT C-5'
R2568D: 5'-CAC TCG CAG CTT GTC GGA TCC GAG CTC CCT CAT TG-3'
3'-GTG AGC GTC GAA CAG CCT AGG CTC GAG GGA GTA AC-5'
E2615R: 5'-GGA AGC TCG TGA GAG ACG TCT GCG GGA GCG GGA G-3'
3'-CCT TCG AGC ACT CTC TGC AGA CGC CCT CGC CCT C-5'
E2638R: 5'-GGC AGC AGG ACC TGC GCA AGG AGC GGG AGG AG-3'
3'-CCG TCG TCC TGG ACG CGT TCC TCG CCC TCC TC-5'
E2670R:5'-CTT GAG AGG GAA CAG AGG CAG CTG CGC CGG GAG-3'
3'-GAA CTC TCC CTT GTC TCC GTC GAC GCG GCC CTC-5'
E2576R: 5'-ATT GAG CAG AGG AAG CAG CGC AGC CTG GAG AAG-3'
3'-GCG CTG CTT CCT CTG CTC AAT GAG GGA GCT CGG-5'
pGEX-KG:5'-GGT GAT CAT GTA ACC CAT CCT G-3'
3'-GTC AGA GGT TTT CAC CGT CAT C-5'

2.1.1 Polymerase Chain Reaction to Create AKAP-Lbc Constructs

The PCR solution for each construct, except for E2576R which used a different procedure, consisted of 5μL of 10x PCR buffer, 1 μL of 2.5 μM dNTPs, 0.8 μL of Pfu Cx Turbo polymerase (Agilent Technologies), 2.5 μL of DMSO, 1 μL of 25 μM AKAP-Lbc parental DNA, and 9.7 μL of ddH_2O. Either 5 μL of the 5 μM construct specific forward primer or 5 μL of the 5 μM construct specific reverse primer were added to the PCR solutions. The samples underwent

PCR conditions at 95°C and 72°C, for a total of 30 cycles. The forward and reverse PCR products of each construct were then mixed and the samples underwent PCR conditions at 95°C and 72°C, for a total of 30 cycles. These constructs were verified by sequencing.

2.1.2 Polymerase Chain Reaction SOEing to Create E2576R Construct

E2576R was generated using the PCR SOEing (sewing by overlap extension) method.[6] Two PCR solutions were made consisting of 5µL of 10x PCR buffer, 1.5 µL of 2.5 µM dNTPs, 1 µL of 25 µM AKAP-Lbc parental DNA, 2.5 µL DMSO, 0.6 µL of Pfu Cx Turbo polymerase (Agilent Technologies), and 29.4 µL ddH_2O. To one PCR sample, 5 µL of 5 µM forward E2576R primer and 5 µL of 5 µM reverse pGEX-kg primer were added. To the other PCR sample, 5 µL of 5 µM reverse E2576R primer and 5 µL of 5 µM forward pGEX-kg primer were added. The samples underwent PCR conditions at 95°C and 72°C, for a total of 30 cycles. A second PCR solution was then made consisting of 5µL of 10x PCR buffer, 1.5 µL of 2.5 µM dNTPs, approximately 10ng of DNA from the PCR products from the E2576R forward pGEX-kg reverse tube, approximately 10ng of DNA from the PCR products from the E2576R reverse pGEX-kg forward tube, 5 µL of 5 µM pGEX-kg forward primer, 5 µL of 5 µM pGEX-kg reverse primer, 0.6 µL of Pfu Cx Turbo polymerase (Agilent Technologies), and 28.4 µL ddH_2O.The samples underwent PCR conditions at 95°C, 52°C, and 72°C, for a total of 28 cycles. This construct was verified by sequencing.

2.2 Protein Expression and Purification

Plasmid vectors pGEX-kg harboring the 257 c-terminal amino acid region of AKAP-Lbc were transformed into BL21-Gold (DE3) *E. coli* cells for the GST protein prep. 2 ml cultures of Luria-Bertani (LB) broth containing ampicillin (Amp) at a concentration of 75 µg/ml were inoculated with singular colonies of the AKAP-Lbc constructs. These cultures were allowed to shake at 220 rpm at 37°C for 12-16 hours. 1.5mL of these cultures were then added to 150 mL LB-ampicillin cultures at the same ampicillin concentration, and were allowed to shake at identical conditions. After 90 minutes, and every 20 minutes thereafter, 0.7mL was removed and absorbance was analyzed at A600 nm value using spectrophotometry. Upon reaching an absorbance between 0.5 and 0.8, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM in each culture. This mixture was then allowed to incubate on the shaker at 220rpm, 37°C for an additional 3 hours. The cultures were then transferred to separate bottles, and were centrifuged at 4°C for 15 minutes at 6000rpm. The supernatants were discarded and the bacterial pellets were placed on ice. Each pellet was homogenized with 2.5 ml of cold GST Buffer A (2.3 M sucrose, 50 mM Tris pH 7.7, 1 mM EDTA) supplemented with protease inhibitors. 10 ml of cold GST Buffer B (50 mM Tris pH 7.7, 10 mM KCl, 1 mM EDTA) supplemented with 1 mM dithiothreitol (DTT) and protease inhibitors was then added to each pellet to homogenize them as well. 4-5 mg lysozyme (MP Biomedicals) was added to each bacterial culture and allowed to incubate on ice for approximately 1 hour, while swirling every 10 minutes. To this mixture, 175 µL 10% sodium deoxycholate, 260 µL 1M MgCl2, and 25µL 5mg/ml DNAse I were added and the tubes was rocked every two minutes by hand until viscosity significantly decreased. They were then centrifuged at 15,000rpm at 4°C for 40 minutes. Meanwhile, glutatione-sepharose beads were combined with ice-cold 14mL T50ED buffer and were suspended by flicking vigorously, followed by centrifuging at 4°C for 3 minutes at 2200 rpm. This supernatant was discarded, and this wash process was repeated two times. The supernatants of the bacterial tubes were then decanted into the glutathione-sepharose bead-containing tubes, and they were allowed to rock for 45 minutes in refrigeration. Next, the tubes were spun for 3 minutes at 1300g, 4°C and the supernatant was decanted. 14mL T50ED buffer supplemented with a concentration of 150 mM NaCl was added to each sepharose pellet and flicked to mix. This was centrifuged at 1300g for 3 minutes at 4C, and the supernatant was discarded. After this wash process was repeated three times, the beads were aliquoted into microfuge tubes (50 µL each) which were then snap frozen in liquid nitrogen, and stored at -80C.

2.3 Co-precipitation Experiments with AKAP and Gα12

To detect interaction between GST AKAP-Lbc lysates and Gα12, co-precipitation assays were performed. The G protein lysates were mixed with $H_{50}E_1D_3M_{10}$ (50mM Hepes, 1mM EDTA, 3mM DTT, 10mM $MgSO_4$) buffer and were dispersed evenly into each interaction tube for which it was needed, with the exception of a load tube which contained 30 µL per protein sample. The GST AKAP fusion lysates also were suspended in HEDM buffer, and 100 µL was cross applied to each of their interaction tubes (containing G proteins/ HEDM), with close attention paid to

preventing cross-contamination. These mixtures were allowed to tilt for 90-120 minutes under refrigerated conditions. The tubes were then centrifuged for 3 minutes at 4000rpm, 4°C. The supernatants were mostly discarded, leaving approximately 20μL fluid in the tube. 1mL ice-cold HEDLM (HEDM plus added 10% LPX at 1/200th volume) was then added to each sample, and the tubes were inverted ten times. The centrifugation and supernatant partial removal was repeated three times. 1M DTT at a ratio of 1:10 with 4x sample buffer both were mixed in a separate tube. 12μL of this mixture was then added to each interaction sample. All tubes were then incubated for 10 minutes at 72°C in a water bath. They were stored at -20°C until polyacrylamide gel electrophoresis was performed. The resulting immunoblots and Coomassie Blue-stained gels were analyzed for binding affinity and equal distribution of GST-fusion proteins between each interaction tube. The antibody used for immunoblotting was rabbit polyclonal antibody to Gα12 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoblot quantitative data was obtained using Kodak GelLogic 100 imaging system and Carestream Molecular Imaging 5.X software to calculate Gaussian fit.

3. Results

To determine whether the charge substitutions interfered with AKAP-Lbc's ability to bind constitutively active (GTP-bound) Gα12, co-precipitation assays were performed. The GTP-bound form was obtained using a mutational strategy involving a glutamine to leucine (Q229L) switch at amino acid 229 (Q229L).[5] A Gα12-specific primary antibody was used to detect binding of the myc-tagged Gα12 in our experiments. Differences in protein expression across all variant protein constructs and the wild type were not found to be significantly different. The only mutants to show a consistent, significant decrease in binding Gα12 were E2582R, R2579E, and E2627R (Fig. 1). The E2629R mutant showed significant decrease in binding as well, but this was inconsistent between immunoblots (Fig. 1).

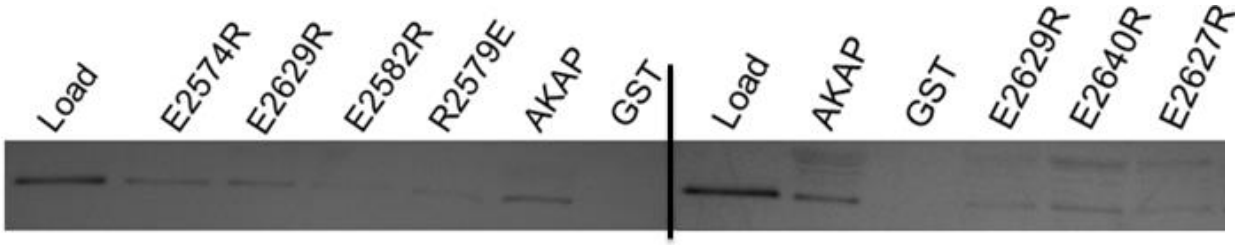

Figure 1. Binding of Gα12 by AKAP-Lbc mutants E2574R, E2629R, E2582R, R2579E, E2640R, and E2627R. The "load" samples serve as a measure of comparative protein abundance. Immunoblots shown are representative of 2 independent experiments.

4. Discussion

Out of the eleven charge substitution constructs made, only three showed consistent, significant decreased binding of Gα12. These constructs were E2582R, R2579E, and E2672R. The fact that these specific residues exhibited impaired binding shows that they are important for AKAP-Lbc to bind Gα12 properly. However, because these constructs do still exhibit some binding of Gα12 and do not show a total inability to bind, it eliminates the possibility that these particular residues are absolutely necessary for AKAP-Lbc to bind Gα12. The result that the specific residues mentioned are important for Gα12 binding suggests that the 106 amino acid region shared by AKAP-Lbc and p114RhoGEF may be important for RhoGEF proteins lacking an RH domain to bind to Gα12. This could be further confirmed by creating constructs of other RH-lacking RhoGEFs that interact with Gα12 at comparable residues. Because AKAP-Lbc's ability to bind Gα12 is potentially correlated with the initiation of pathologic cardiac hypertrophy, mutations in this protein which may impair its ability to bind Gα12 could have medical implications.[2] Further work is needed to better characterize how the glutamic acid residues at 2582 and 2672 and the arginine residue at 2579 are involved in the interaction between AKAP-Lbc and Gα12 and also to determine what other residues may be important Gα12 binding.

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6. References

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