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Domain Replacement in Galpha12 by an Evolutionary Homolog Reveals Key Residues in Cell Growth Signaling

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Abstract

The G12 class of heterotrimeric G protein α subunits is important for several cellular processes that include mitogenesis, cytoskeletal rearrangements, and migration. Previous work in the Meigs lab revealed that an evolutionary G12 homolog from *Drosophila*, termed Concertina, lacked the ability of mammalian G12 proteins to stimulate a growth signaling pathway in cells. Therefore, several chimeras of Gα12 and Concertina were engineered, and these provided evidence that the C-terminal domain of Gα12 is essential for activating serum response element (SRE) mediated transcription. To dissect this large C-terminal region, overlapping PCR was used to engineer a SRE-coupled chimera that included an additional 45-residue Concertina domain in the middle of its C-terminal region. This modification completely uncoupled the chimeric protein from SRE-mediated transcription as demonstrated by a firefly luciferase reporter gene assay. The N-terminal Concertina domain of this chimera was then replaced with Gα12 sequence, and it was demonstrated that this 45-residue Concertina substitution in the C-terminus was sufficient to uncouple Gα12 from SRE signaling. This new chimera can now be used to more precisely define the determinants of growth signaling activation within Gα12.

1. Introduction

Living cells have the basic ability to perceive and respond to their environment using transmembrane protein receptors to trigger cellular responses to stimuli. G-protein coupled receptors (GPCRs) are a conserved family of transmembrane receptors which detect a wide range of stimuli including neurotransmitters, hormones, light and odorants.[1] Activated GPCRs undergo a conformational change that allows them to activate heterotrimeric G proteins.[1] Each G protein has an α, β and γ subunit which are tightly bound in an inactive state when the α subunit is bound to GDP.[1] Activation of G proteins by GPCRs causes Gα to exchange GDP for GTP, dissociate from the G$\beta\gamma$ heterodimer and activate specific downstream effector molecules based on the class of the Gα protein.[1] Gs and Gi signal by stimulating and inhibiting adenylyl cyclase, Gq subunits signal through phospholipase C and G12/13 subunits activate the serum response element (SRE) transcription pathway and stimulate cellular contractility through the activation of Rho-specific guanine nucleotide exchange factors.[1]

The G12/13 class of G proteins has been shown to regulate several key signaling networks controlling cell migration, cytoskeletal rearrangements, adhesion, apoptosis, and mitogenesis often by modulating SRE mediated transcription[1]. Overexpressed or mutationally active G12/13 proteins are implicated in oncogenic transformation and endogenous levels of $G\alpha_{12}$ in breast cancer tissue correlate to the degree of advancement towards metastasis.[1,2,3] Mutating the glutamine at position 229 to a leucine creates a mutationally activated form called $G\alpha_{12}^{QL}$.[2] This mutation is useful for experimentation because it drives signaling pathways such as SRE at a high rate. Further mutations can then be made to compare the strength of the signal response to normal $G\alpha_{12}^{QL}$.

A recent study dissected the contributions of the N-terminal, Switch regions, and C-terminal domains of Gα_{12} to downstream signal activation by replacing these domains with the homologous regions from the ancestral G12/13 homolog in *Drosophila melanogaster* termed Concertina (Cta).[1,4] Cta is required for cellular shape changes and migration and shares ~55% amino acid identity with Gα_{12} but it does not activate the SRE pathway.[1] The Gα_{12}/Cta chimera proteins revealed that the Switch regions and C-terminal domain of Gα_{12} contain distinct features not found in Cta which are necessary for activating SRE-mediated transcription.[1] Replacing either the Switch regions or the C-terminus with Cta completely uncoupled the chimeric protein from SRE activation.[1] The chimera containing a Cta N-terminal domain with Gα_{12} Switch regions and C-terminal domain was shown to retain SRE activation functioning so I decided to further modify this chimera to contain a 45-residue Cta substitution within the C-terminus in order to dissect the large domain.[1] The area designated for substitution is shown in Figure 1 and was selected because of the relatively low amount of conservation in the region between Gα_{12} and Cta. Figure 2 shows the oligonucleotides used for the sewing PCR. I demonstrated that the new chimera was completely uncoupled from SRE-mediated transcription through a firefly luciferase reporter gene assay. Then, I replaced the N-terminal Cta domain with the original Gα_{12} N-terminus and was able to demonstrate that the 45 amino acid mutation in the C-terminus was sufficient to completely uncouple the protein from SRE activation.

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            230        240        250        260        270        280
Gα12    VDVGGQRSQRQKWFQCFDG-ITSILFMVSSSEYDQVLMEDRRTNRLVESMNIFETIVNNK
        :::::::::::::: .::::. .::::.:.::::::.:::: :::.:::: :: :::.:::::
Cta     VDVGGQRTQRQKWTRCFDSSVTSIIFLVSSSEFDQVLAEDRKTNRLEESKNIFDTIVNNA
        300        310        320        330        340        350

             290        300          310        320        330        340
Gα12    LFFNVSIILFLNKMDLLVEKVKS--VSIKKHFPDFKGDPHRLEDVQRYLVQCFDRKRRNR
         : ..::::::::: ::: .:: .  ..:. ..: :.:.:: . ::: ...: :    ::.
Cta     TFKGISIILFLNKTDLLEQKVCNPETDIRWYYPHFNGNPHSVLDVQNFILQMFMSVRRSS
        360        370        380        390        400        410

                 350        360        370
Gα12    S-KPLFHHFTTAIDTENIRFVFHAVKDTILQENLKDIMLQ
        : . ..:::::::::.::  ::..:::::::.::. .:::
Cta     SISRIYHHFTTAIDTRNINVVFNSVKDTILQRNLNALMLQ
        420        430        440        450
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Figure 1. Comparison of the C-terminal domains of Cta and Gα_{12}. The underlined region is the C-terminal domain and the highlighted region is the area of low-conservation selected for substitution. Two vertical dots indicate an exact residue match, one dot indicates similarity and no dot indicates non-conserved residues.

sewing PCR oligos

5'-AGGATTGCACACCTTCTCCACCAGGAGGTC-3' (rv oligo at the 1st junction)
5'-AGGATCTACCACCACTTCACCACCGCCATAG-3' (fw oligo at the 2nd junction)
5'-GAGAAGGTGTGCAATCCTGAGACTGACATTC-3' (fw oligo at the 1st junction)
5'-GAAGTGGTGGTAGATCCTACTTATGCTAC-3' (rv oligo at the 2nd junction)

pcDNA 3.1 (+) oligos

Starting at BP 828 5'-AACTAGAGAACCCACTGCTTAC-3'

Comp. to BP 1101 3'- GAGGGTGACAGGAAAGGATTA-5'

Figure 2. The oligonucleotides used for sewing PCR with the region base-pairing the original chimera highlighted yellow. The pink matches the region of Cta to be inserted into the chimera. The pcDNA 3.1(+) oligonucleotides were used in the second round of PCR to replicate the entire construct.

2. Methods

2.1 DNA Constructs

The original chimera protein (chimera 4) consisting of myc-Gα_{12}^{QL} with a Cta N-terminal domain was previously produced in our lab in the vector pcDNA 3.1 (+) (Invitrogen).[1] The 45 amino acid substitution in the C-terminal domain was then engineered using a two-step PCR process. The region of Cta to be substituted was amplified using oligonucleotides which were designed to contain an additional nine base-pairs that overlapped with the amplified region on the original chimeric protein. The regions upstream and downstream of the substitution on the original chimera were also amplified using oligonucleotides which had an additional nine base-pairs that matched the amplified region on Cta. This technique created three amplified regions with an area of overlap of 18 base-pairs at each of the two junctions. The amplified regions were then combined and subjected to a second round of PCR which amplified the entire construct. The new construct (subchimera) was then ligated back into pcDNA3.1(+) (Invitrogen), verified by sequencing and assayed for SRE activation. The construct was then modified to replace the Cta N-terminal domain with the Gα_{12} N-terminus using the restriction enzymes *Eco*NI and *Nhe*I to cut both the construct and myc-Gα_{12}^{QL} in pcDNA3.1(-). The products were separated by gel electrophoresis and the myc-Gα_{12}^{QL} N-terminal fragment was used as the insert and ligated onto the chimera contained within pcDNA3.1(+). The final product was myc-Gα_{12}^{QL} with a 45 residue Cta substitution contained in the middle of the C-terminus (subchimera 2).

2.2. Reporter gene assays

The chimeric protein constructs were transformed into HEK293 cells grown in 12-well plates. The cells were also transfected with 0.2µg SRE-luciferase plasmid (encoding firefly luciferase) provided by Channing Der (UNC-Chapel Hill) and 0.02µg pRL-TK (encoding *Renilla* luciferase; Promega).[1] Cells were washed with phosphate-buffered saline and lysed using 1X passive lysis buffer (Promega).[1] Lysates were analyzed using a Dual-luciferase assay program on a GloMax 20/20 luminometer (Promega).[1] The ratio of light output due to firefly luciferase activity to light output from *Renilla* luciferase was taken to normalize for transfection efficiency.[1]

2.3. SDS-PAGE and Western blot analysis

Polyacrylamide gel electrophoresis (PAGE) followed by a Western blot was performed after each reporter gene assay. The gel was 12% Protogel with SDS as the detergent and Benchmark prestain protein ladder. The Western blots were performed overnight at 35V and 4°C. The blots were chilled overnight in 5% bovine milk TBST solution and 1:1000 Millipore anti-myc mouse antibody solution. One blot (Figure 4B) was placed in the same conditions except for using a 1:1000 primary rabbit antibody specific to Gα_{12} (Santa Cruz Biotechnology). The blots were washed with TBST and secondary antibodies specific to mouse or rabbit respectively were added in a ratio of 1:7500 for at least 1hr. The blots were washed in TBST again then developed until visible bands appeared.

3. Results

The results summarized in Figure 3 show that the 45-residue substitution caused a significant decrease in the light output due to firefly luciferase. Since the firefly luciferase was transcribed by the SRE pathway, the substitution was demonstrated to be sufficient to cause uncoupling of the protein from the SRE pathway. The proteins were also shown to be expressed in approximately equal proportions as shown by the immunoblot bands at ~54kDa (Figure 4).

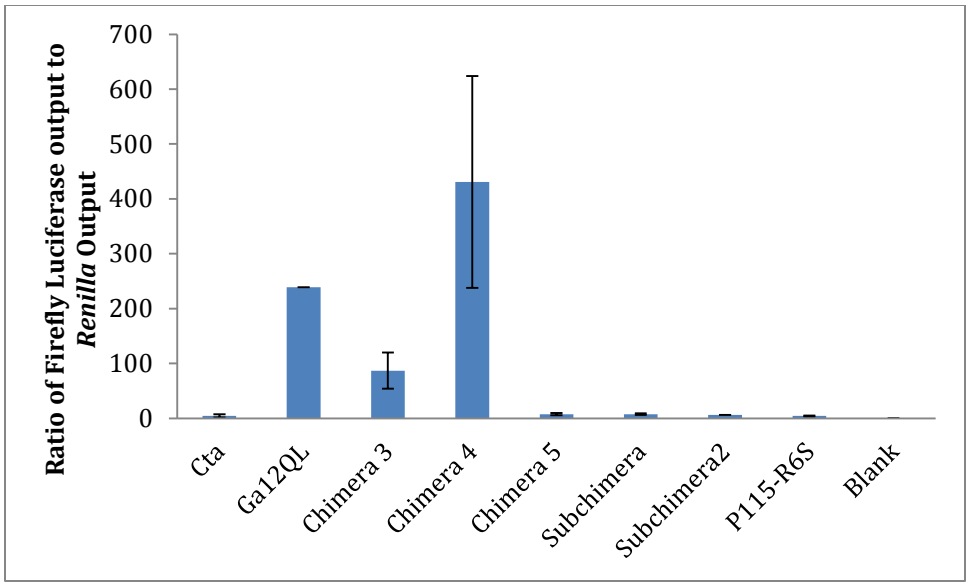
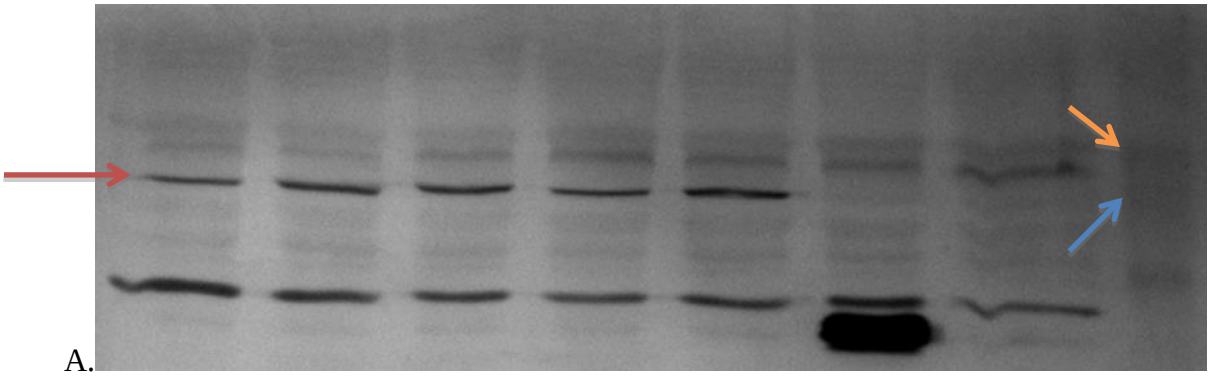


Figure 3. The average results of firefly luciferase reporter gene assay. The ratio of light output due to firefly luciferase activity to light output from *Renilla* luciferase was taken to normalize for transfection efficiency. Numbered chimeras were previously made in the lab and confirm the previous results that only chimera 4 activates SRE. Subchimera and Subchimera 2 contain the 45-residue Cta substitution and demonstrate that it uncouples the protein from SRE activation. P115-R6S acted as a DNA control. The error bars represent one standard deviation.

A.



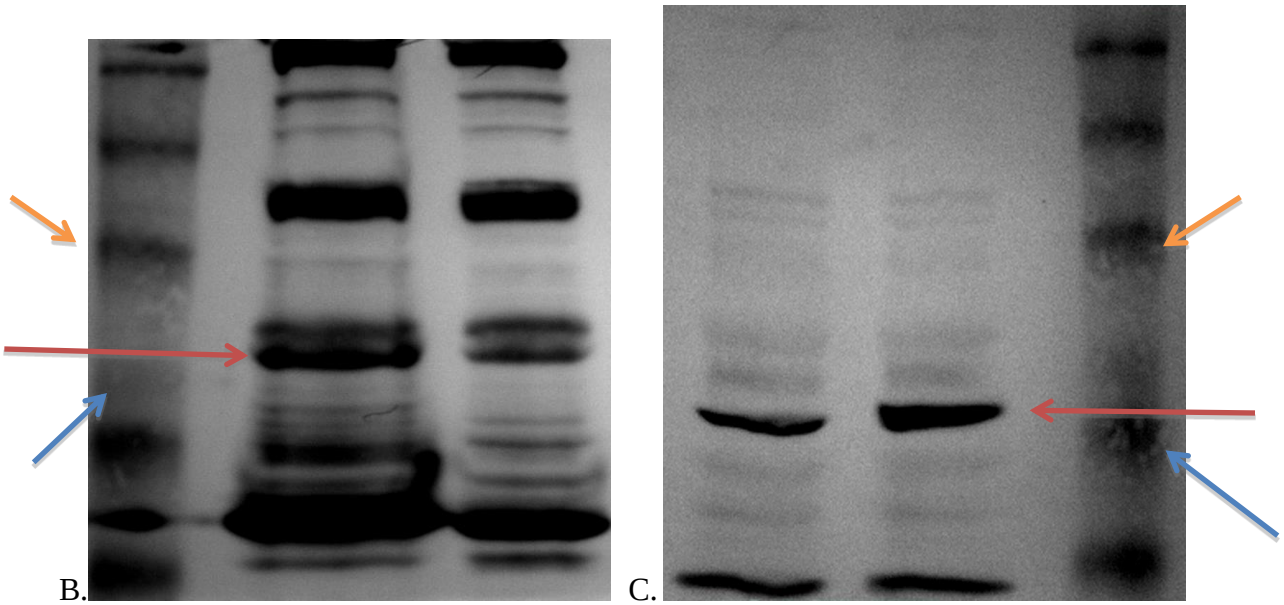


Figure 4. Developed blots showing that the proteins were expressed for the reporter gene assays. Red arrow indicates location of proteins of interest. Orange arrow indicates 64kDa band, blue indicates the 49kDa band. A: Cta, Chimera 3, Chimera 4, Chimera 5, Subchimera, P115-R6S (control), Blank, Ladder; B: Ladder, $G\alpha_{12}^{QL}$, Subchimera 2; C: Chimera 4, Subchimera, Ladder

4. Discussion

The results show that within the 42-residue C-terminal region of $G\alpha_{12}$ is a necessary component of G protein activated SRE mediated transcription. It is key that the replacement was from the evolutionarily related protein Cta and that no other mutation was necessary to shut off SRE. Cta shares ~55% amino acid identity with $G\alpha_{12}$ and, similarly to $G\alpha_{12}$, it has been shown to stimulate the Rho-dependent processes of cytoskeletal rearrangements and contractility in cultured *Drosophila* cells by interacting with a RhoGEF (DRhoGEF2) which is very closely related to mammalian G12/13-coupled RhoGEFs.[1] Since the proteins are so closely related it was surprising that such small changes in their primary structure affect their signal pathway activation capabilities so significantly. Future studies could further dissect the region and change specific residues that vary between Cta and Gα12

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