

Comparative Interaction of Cadherin Cell Adhesion Proteins with Guanine Nucleotide-Binding Proteins $\text{G}\alpha 12$ and $\text{G}\alpha 13$

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Abstract

Cell migration, proliferation, and attachment are critical processes for both normal tissue growth, and the development of malignant disease. The G12/13 class of heterotrimeric guanine nucleotide-binding (G) proteins and their interaction with cadherin cell adhesion proteins has been implicated in the regulation of these processes. The two members of the G12/13 class, $\text{G}\alpha 12$ and $\text{G}\alpha 13$ are structurally similar, sharing 67% amino acid homology, but show only partial overlap in effector signaling.¹ Previous work demonstrated that $\text{G}\alpha 12$ binds an acidic 11 amino acid region of the cytoplasmic C-terminal domain of epithelial (E)-cadherin, a membrane spanning cell adhesion protein responsible for adherens junction formation. This interaction was shown to cause cell disassociation and cell migration,² a hallmark of metastasis. While this region of E-cadherin was implicated in this binding event, the interacting residues and affinities of $\text{G}\alpha 12$ and $\text{G}\alpha 13$ with E-cadherin and other cadherin family members have yet to be elucidated. Here, epitope tagged mutants of $\text{G}\alpha 12$ and chimeric constructs of $\text{G}\alpha 12$ and $\text{G}\alpha 13$ were used to examine binding affinities of these proteins with several members of the cadherin family. Interestingly, the activated form of $\text{G}\alpha 12$ had a higher affinity for all cadherins tested when compared to $\text{G}\alpha 13$, suggesting $\text{G}\alpha 12$ may play a larger role in the regulation of these cell adhesion proteins. Pursuant to previous investigations, six sextet cassette mutants of eGFP tagged activated $\text{G}\alpha 12^{\text{QL}}$ spanning the switch region I were utilized to examine binding to E-cadherin and VE-cadherin, which surprisingly failed to uncouple interaction. The E-cadherin deletion mutant that lacks the 11 amino acid region and had been demonstrated to disrupt binding of $\text{G}\alpha 12^2$ was tested alongside $\text{G}\alpha 13$ and strikingly showed strong binding to $\text{G}\alpha 12^{\text{QL}}$ but abrogated binding to $\text{G}\alpha 13^{\text{QL}}$. These data show that a potentially different activational conformation exists between $\text{G}\alpha 12^{\text{QL}}$ and $\text{G}\alpha 13^{\text{QL}}$, but that both G proteins bind a common region of the cytoplasmic domain of E-cadherin.

1. Introduction

Heterotrimeric guanine nucleotide-binding proteins (G proteins) mediate numerous cellular physiological pathways, including cell proliferation, cytoskeletal changes, cell growth, and oncogenic transformation. These proteins are composed of a tightly associated $\beta\gamma$ dimer and a α subunit, whose activation is dependent on GDP-GTP exchange. Upon stimulation of the G protein's G protein coupled receptor (GPCR), the α subunit binds GTP and disassociates from the $\beta\gamma$ dimer, allowing it to interact with its downstream effectors.¹ Recent studies have shown that $\text{G}\alpha 12$ and $\text{G}\alpha 13$ are unique among the G protein subfamilies in that overexpression, and not mutational activation, of these proteins drives neoplastic transformation in cells, making these molecules excellent targets of research for cancer therapies.^{1,3,4}

Classical cadherins are a large family of single-pass transmembrane proteins that mediate cell adhesion through the formation of adherens junctions.⁵ Cadherins consist of an extracellular N-terminal domain and a cytoplasmic C-

terminal domain. The extracellular domain binds homotypically with cadherins of the same isoform in a Ca^{2+} dependent manner, and it is this homotypic binding of cadherin types that is largely responsible for the demarcation seen between discrete tissues.⁵ The cytoplasmic domain of cadherins have several binding partners necessary for the structural integrity of the protein and maintenance of the cytoskeleton, including α -catenin, β -catenin, and p120-catenin, with α -catenin serving to bind the actin cytoskeleton, stabilizing the cytoskeleton at the adherens junction. In addition to facilitating cellular adhesion, there is evidence that E-cadherin acts as a tumor suppressor.⁶ Many cancers of epithelial tissue show a marked decrease in E-cadherin expression, the principle cadherin of epithelial cells. This decreased expression is a hallmark of highly invasive cancers, while a higher expression of E-cadherin correlates to less invasive neoplastic tissue.⁶ As mentioned, the cytoplasmic domain also serves to bind the transcriptional co-activator β -catenin, which has been demonstrated to activate several oncogenes once inside the nucleus.⁷

Previous investigation has demonstrated that $\text{G}\alpha 12$ bound to an acidic 11 amino acid sequence of the E-cadherin cytoplasmic tail and caused cell migration and the dislocation of β -catenin from the cytoplasmic tail complex to the cytoplasm.² While this revealed a region of E-cadherin implicated in binding $\text{G}\alpha 12$, the surfaces of $\text{G}\alpha 12$ responsible for this interaction were not studied. Recently, it was found that $\text{G}\alpha 13$ interacts with the cytoplasmic tail of vascular endothelial (VE)-cadherin at a cytoplasmic tail sequence of high similarity to that of E-cadherin by $\text{G}\alpha 12$, and that it is the switch region I of $\text{G}\alpha 13$ that binds to this distinct region on VE-cadherin.⁸ The similarity of $\text{G}\alpha 12$ and $\text{G}\alpha 13$ in structure and the structural homology between E-cadherin and VE-cadherin, served as the impetus to determine if the switch region I of $\text{G}\alpha 12$ is responsible for E-cadherin interaction. A comparative interaction study of $\text{G}\alpha 12$ and $\text{G}\alpha 13$ with E-cadherin, VE-cadherin, N-cadherin, and Cadherin-14 was also undertaken to better understand the binding affinities between these G proteins and several members of the cadherin family, using eGFP tagged forms of the G proteins. These interactions were also tested with eGFP tagged chimeras of $\text{G}\alpha 12$ and $\text{G}\alpha 13$ to more accurately define the regions of each G protein responsible for interaction. Finally, because $\text{G}\alpha 13$ was not examined in relation to the acidic 11 amino acid sequence of E-cadherin, eGFP tagged $\text{G}\alpha 13^{\text{QL}}$ was employed in co-precipitation assays with this E-cadherin deletion mutant.

2. Materials and Methods

2.1 Epitope Tagging of $\text{G}\alpha 12$ Constructs

2.1.1 GFP Tagging of $\text{G}\alpha 12$ NAAIRS Mutants

All NAAIRS mutants had previously been tagged with a myc epitope (amino acid sequence: EQKLISEEDL) with SGGGGS linkers by a silent restriction site (AgeI) at residues P¹³⁹ V¹⁴⁰ and were housed in pcDNA 3.1 (-). NAAIRS mutants designated FF, GG, HH, II, JJ, KK were digested with AgeI HF (New England Biolabs) at 37°C for 1 hour alongside a pcDNA 3.1 (-) plasmid containing eGFP. After 1 hour, 1.5 μ L of calf intestinal phosphatase (New England Biolabs) was added to the NAAIRS mutant plasmids and allowed to continue at 37°C for 1 hour. Gel electrophoresis was used to separate the resultant DNA on 1% (w/v) agarose and the NAAIRS mutant plasmids and the GFP insert were excised and isolated from the gel by mini column (Promega Wizard Kit). 500ng of plasmid was then ligated with 9 μ L of eGFP insert DNA using T4 DNA ligase (New England Biolabs) for 1 hour at room temperature then overnight at 4°C. JM109 competent cells were transformed with 1 μ L of the respective ligations and were grown on 100 μ g/mL ampicillin agar plates for 12-16 hours. Resulting singular colonies were picked and grown in 75 μ g/mL ampicillin Luria Broth (LB) for 12 hours. DNA was isolated from cells by mini column (Qiagen) and quantified by spectroscopy at 260nm. DNA was then transfected into HEK293 cells and examined under fluorescent microscopy for stable GFP expression.

2.1.2 GFP and Myc tagging of $\text{G}\alpha 12/\text{G}\alpha 13$ Chimeras

A silent mutation producing an AgeI cut site was introduced into each of the four chimeric constructs by polymerase chain reaction (PCR) using the following oligonucleotides:

FW – GAACAAGGCAGGGCTACCGGTGGAGCCTGCCACC
RV – GGTGGCAGGCTCCACCGGTAGCCCTGCCTTGTTC

Two separate reactions and cycles of forward alone oligonucleotide and reverse alone oligonucleotide were performed at 95°C for 35 seconds, 54°C for 1 minute, and 68°C for 12 minutes, before being combined and continued for an additional 15 cycles at the same conditions. Reactions were then digested with DpnI for 1 hour to eliminate parent DNA present in the reaction and these samples transformed into JM109 cells for DNA propagation. Presence of successful AgeI site insertion was determined by diagnostic restriction enzyme digest and subsequent gel electrophoresis. Splicing of eGFP epitope tag was then performed as described above.

Because the DNA sequence for the myc epitope tag was too small to be visualized and gel excised, phosphorylated oligonucleotides

(FW) [PHOS]CCGGTGAGCGGAGGAGGAAGCGAGCAGAAGTTAACGCGAAGAAGATTAAAGCGGAGG
AGGAGGATCA / RV
[PHOS]CCGGTGATCCTCCTCCGCTTAAATCTTCTCGCTGATTAACCTCTGCTCGCTCCTCCTCCTC
CGCTCA) were used to produce the double stranded DNA encoding the myc tag sequence. Both oligonucleotides were combined at 1 μ M in a microfuge tube using elution buffer (Qiagen) as the diluent and heated for 5 minutes at 95°C. Approximately 1.5L of water was brought to 85°C and the annealing reaction was placed in the water bath and the beaker removed from heat. The reaction continued for 2 hours, until the water bath reached 40°C. This myc DNA sequence was then ligated into the chimeras using the methods previously described.

2.2 Cloning of G α 12/G α 13 Chimeras into pcDNA 3.1(-)

Chimeras of G α 12 and G α 13 were generously donated by Dr. Barry Kreutz of the University of Illinois at Chicago. To facilitate the eGFP epitope tagging of these constructs, it was necessary to move them to pcDNA 3.1(-) to avoid extra AgeI cut sites in pCMV that originally housed the chimera DNA and would have interfered with the ligation process. Two different sets of forward and reverse primers were required for PCR to amplify the chimeras due to their ends being either G α 12 or G α 13 sequence. Also, BamHI sites and KpnI sites were inserted in the forward and reverse oligonucleotides, respectively, to enable cloning of these chimeras into pcDNA 3.1 (-). Cut sites are underlined.

Chimeras #1 & #2: FW 5'-GTTGGGATCCACCACCATGTCCCGGGTGGTGCGGACC- 3'
RV 5'-GTTGGGTACCTCACTGCAGCATGAGCTGCTTCAG- 3'

Chimeras #3 & #4: FW 5'- GTTGGGATCCACCACCATGGCGGACTTCCTGCCGTCG- 3'
RV 5'- GTTGGGTACCTCACTGCAGCATGATGTCTTC- 3'

The PCR conditions for the amplification of the chimeras were as follows: 95°C for 2 min, 94°C for 45 sec, 53°C for 1 min, 72°C for 2.5 min at 27 cycles, followed by a final 72°C 5 min elongation step. PCR products were then ligated into pcDNA3.1 (-) as previously described and successful ligation was confirmed by diagnostic restriction digest.

2.3 Creation of GST-VE-cadherin C-terminal tail

Full length VE-cadherin gene sequence was purchased from GE healthcare in bacterial glycerol stock. Oligonucleotides were designed to amplify the DNA sequence representing the 98 C-terminal amino acids of the VE-cadherin gene. A forward primer BamHI site and a reverse primer EcoRI site were inserted to facilitate the ligation of the PCR product into the GST harboring pGEX-KG plasmid (GE healthcare). Below are the amplifying primers:

FW: 5'- CGATGGATCCCCGAGGCACGCGCCTGGGGCAC- 3'
RV: 5'- TAGCGAATTCTAATACAGCAGCTCTCCCG- 3'

PCR conditions used were as described in section 2.2.

2.4 Expression and Purification of GST fused proteins

Vectors containing the GST-cadherin cytoplasmic tail sequences were transformed into BL21(Gold)DE3 *E. coli*. Individual respective colonies for each GST construct were then inoculated in 12mL of Luria Broth (LB) with ampicillin (amp) added to a concentration of 75 μ g/mL. These cultures were then grown for 12-16 hours at 37°C under 220rpm agitation. After this growth phase, 5mL of this turbid culture was then inoculated in 500mL of LB under the same growth and amp concentration conditions. After 90 min, this 500mL culture was sampled and its concentration measured by spectrophotometry at 600nm. When the A₆₀₀ value was between 0.5 and 0.8, induction of protein expression was stimulated by isopropyl- β -D-thiogalactopyranoside addition and the culture incubated for 3 hours. Bacteria were collected by centrifugation at 6000 x g at 4°C for 15 min. These cells were then homogenized in 5.5mL of cold GST Buffer A (2.3M sucrose, 50mM Tris pH 7.7, 1mM EDTA) containing a 1:500 dilution of protease inhibitors. 10mL of cold GST buffer B (50mM Tris pH 7.7, 10mM KCl, 1mM EDTA, 1mM DTT, 1:500 protease inhibitors) was then added to the homogenized bacterial sample and mixed. Following a 4-5mg lysozyme addition, the homogenized bacterial sample was incubated on ice for approximately 1 hour and mixed every 10 min. Following this incubation, 175 μ L of 10% sodium deoxycholate, 260 μ L of 1M MgCl₂, and 25 μ L of 5mg/mL DNase I was added to the crude lysate and further incubated on ice for approximately 10 min. This mixture was rocked by hand every 2 min until a noticeable loss of viscosity was observed. This mixture was then refined by centrifugation at 15,000 x g, 4°C for 40 min and the resulting supernatant was added to 0.35mL of glutathione-sepharose beads (GE healthcare) that had been previously washed in T₅₀ED buffer (50mM Tris pH7.7, 1mM EDTA, 1mM DTT). This supernatant and glutathione-sepharose was machine rocked for 45 min at 4°C to allow interaction. The GST immobilized proteins were then washed further with T₅₀ED buffer supplemented with 150mM NaCl four times and aliquots of these purified proteins were snap frozen in liquid N₂.

2.5 Preparation of G α 12 and G α 13 constructs from whole cell lysates

Human embryonic kidney (HEK293) cells were cultured in Dulbecco Eagle medium with 10% fetal bovine serum and antibiotics, incubated in a 5% CO₂ atmosphere at 37°C. Approximately 10 μ g of construct DNA and 2mg/mL of polyethylenimine (PEI) were used to transfect HEK293 cells in a 10cm dish, with cells at 90% confluence. DNA and PEI were mixed with 500 μ L of DMEM and incubated for 15 min at room temperature, then introduced drop-wise to the cell plates. At 36-48 hours post transfection, the plates were rinsed with 4°C sterile phosphate buffered saline (PBS), scraped and collected in 3mL of PBS by centrifugation at 500 x g at 4°C. Each cell pellet then received 500 μ L of ice-cold lysis buffer (50mM HEPES pH 7.5, 1mM EDTA, 3mM DTT, 10mM MgSO₄, 1% polyoxyethylene-10-lauryl ether (LPX), 50% of volume MP 2X protease inhibitor mix, 1:500 protease inhibitor mix) and cells were lysed on ice by trituration. Samples were mixed by inversion at 4°C for 30 min, and then centrifuged at approximately 64,000 x g 3°C for 1 hour. Supernatants were then collected and 60 μ L aliquots of each construct were snap frozen in liquid N₂.

2.6 Co-precipitation of GST-cadherins and G α 12/13 constructs

Each 60 μ L lysate containing the G α 12/13 construct to be assayed was diluted with HEDM buffer (50mM HEPES pH 7.5, 1mM EDTA, 3mM DTT, 10mM MgSO₄) to lower the LPX concentration to approximately 0.075%. From this dilution, 40 μ L was removed to serve as the load control for the lysate being assayed, and the remainder equally distributed to be incubated with the GST-cadherin construct. These samples were allowed to interact for approximately 3 hours at 4°C by inversion. Interaction tubes were then centrifuged at 1300 x g at 2°C and the supernatants removed. Pelleted GST-sepharose beads were then washed 3 times with 1mL of HEDM buffer containing LPX at a concentration of 0.05%. Denaturation was achieved by addition of SDS sample buffer supplemented with 10% DTT at 72°C for approximately 10 min. Denatured proteins were then separated by SDS-PAGE, with 3 μ L of each sample used as the loading control for the Coomassie Blue stain gel, to determine equal amounts of GST-fused cadherin available in each *in vitro* interaction condition. Remaining protein was separated by SDS-PAGE and transferred to nitrocellulose paper for Western blotting. eGFP primary antibody (Thermo Scientific) was diluted to 1:1000 in 5% milk and TBST (Tris buffered saline, Tween-20) solution and incubated with immunoblot for 3 hours at room temperature with agitation. Following three 10-minute washes of the immunoblot with plain TBST, a 1:7500 dilution of alkaline phosphatase conjugated secondary mouse antibody (Promega) in 5% milk and

TBST was introduced and incubated for 1 hour under constant agitation. Three additional TBST washes were followed by detection of protein bands colorimetrically using NBT/BCIP in alkaline phosphatase buffer solution. Resulting blots were quenched with ddH₂O and photographed using Kodak GelLogic 100 imaging system and Gaussian fit densitometric data was obtained by Carestream 5X software.

3. Results

The highly similar amino acid homology between G α 12 and G α 13 led to the question of whether binding affinities with the cadherin types differed between G α 12 and G α 13. In order to facilitate the detection of both G proteins with immunoblot analysis, without the need for using two specific antibodies, a common epitope tag was needed. The eGFP (enhanced green fluorescent protein) tag was chosen because it adds approximately 26kDa of mass to the G proteins, allowing the epitope tagged protein to be easily distinguishable from endogenous G α 12 and G α 13 proteins from whole cell lysates (Fig. 1A). eGFP is also beneficial because it can be inserted into the alpha helical domain of both G proteins and does not disrupt the functional conformations of either protein, as evidenced by standard SRE luciferase response⁹, of which G α 12 and G α 13 are potent stimulators (Fig. 1B). In addition, eGFP tagged G α 12 and G α 13 can be visualized by fluorescent microscopy (Fig. 1C).

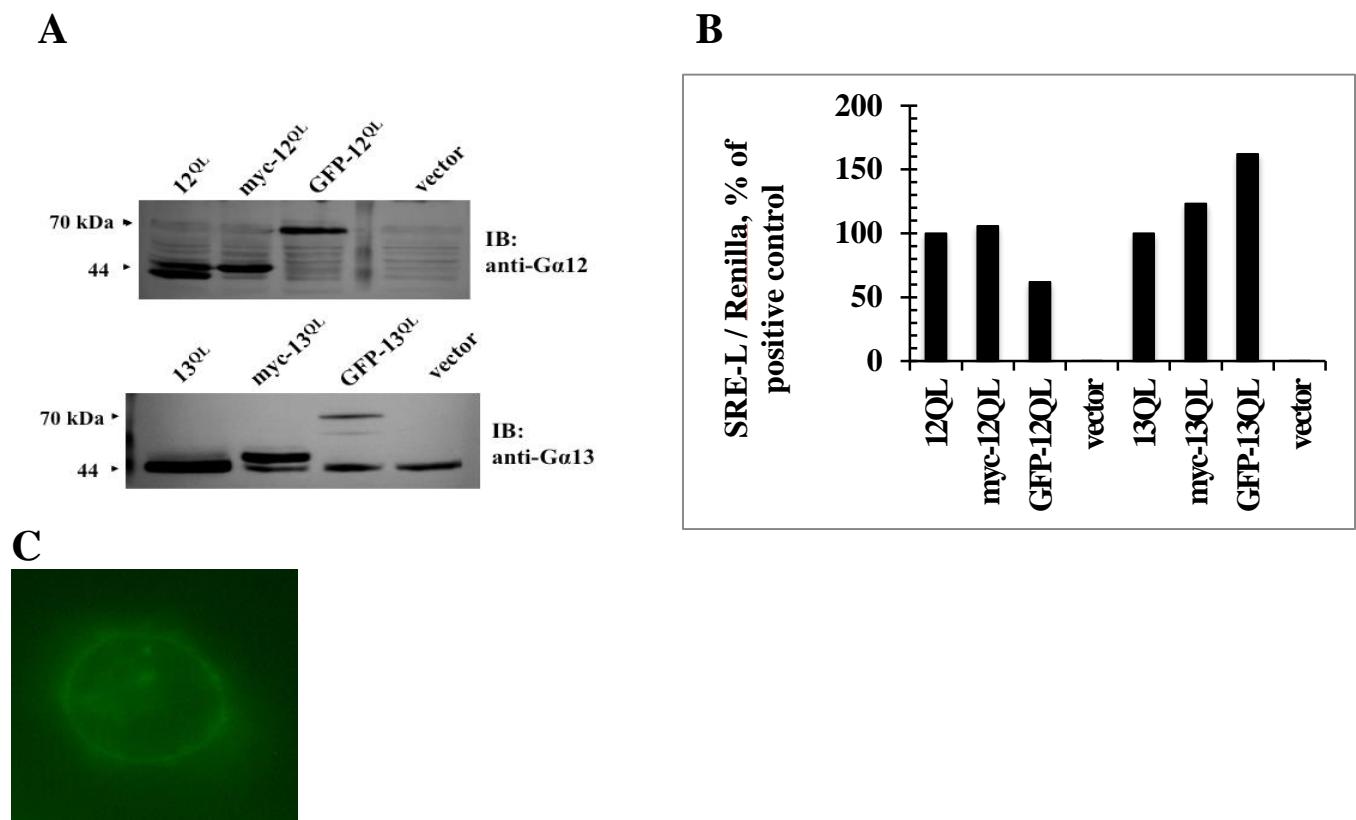


Figure 1. eGFP tagged G α 12 and G α 13 retain functionality. (1A) Immunoblot demonstrating the increased molecular weight of eGFP tagged G α 12 and G α 13 using antibodies for both G α 12 and G α 13. The myc tag is another epitope tag that is commonly used but was chosen against due to poor immunoblot detection. (1B) SRE/Renilla response assay of epitope tagged G α 12 and G α 13 along with untagged protein. This assay is highly robust and regularly evidences proper folding conformation of both proteins. (1C) HEK293 cell showing eGFP tagged G α 12 within the cell.

To further interrogate the findings that the switch region I of Gα13 is necessary for the interaction with VE-cadherin⁸, constitutively activated NAAIRS cassette substitution mutants of Gα12 spanning the switch region I were implemented in co-precipitation assays with E-cadherin and VE-cadherin. These cassette substitution mutants were utilized due to their highly tolerated sequence of asn-ala-ala-ile-arg-ser (NAAIRS) within the conformational structure of Gα12 (Fig.2). Surprisingly, no switch region I spanning NAAIRS mutant failed to uncouple binding to the 98 C-terminal amino acids of either E-cadherin (Fig. 3A,B) or VE-cadherin (data not shown) in co-precipitation assays, with precipitate to load ratios all greater than 1, demonstrating strong interaction. These results were particularly surprising due to the high sequence homology seen between the switch region I of Gα12 and Gα13.

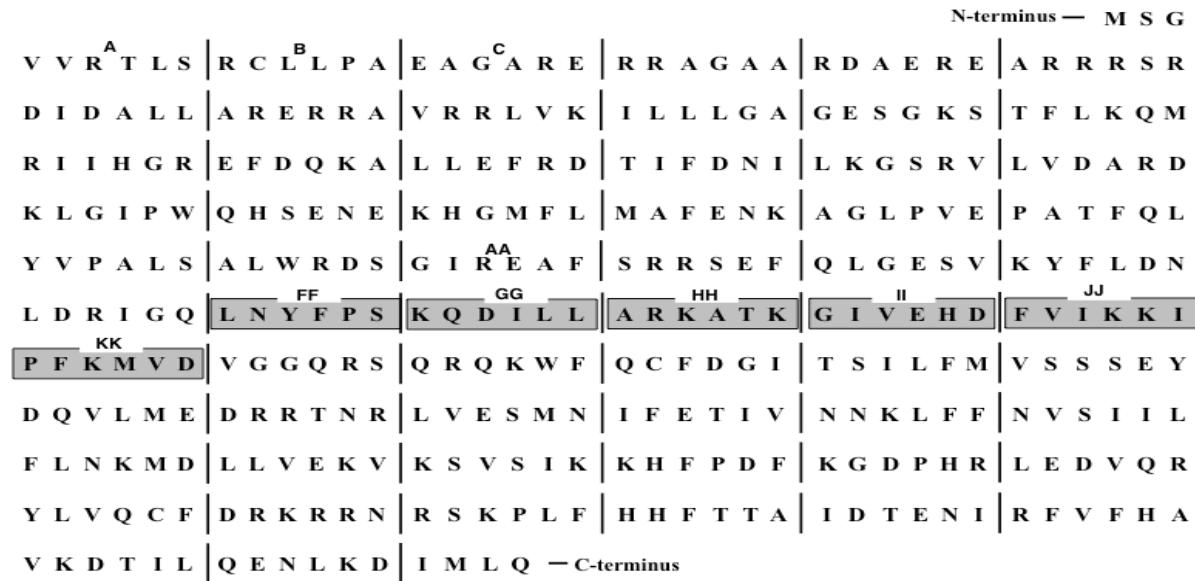


Figure 2. Highlighted boxes show the regions of primary Gα12 sequence that are substituted with the NAAIRS mutant sequence. The actual switch region I is composed of the ARKATKGIVEH sequence, within the HH and II NAAIRS mutants. The double lettered alphabetical nomenclature system designates the specific NAAIRS mutant.

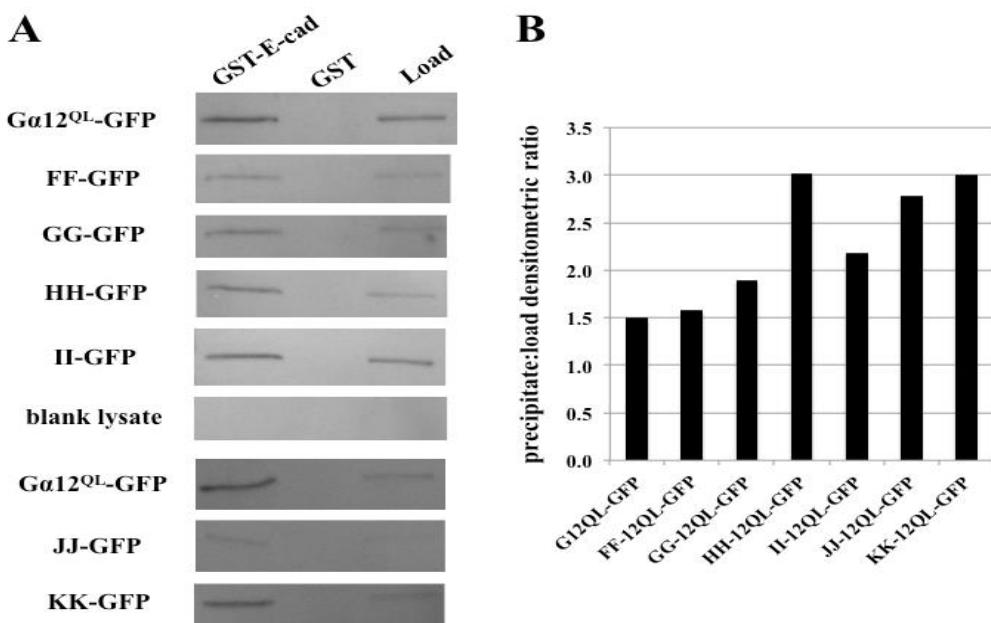


Figure 3. No $\text{G}\alpha 12^{\text{QL}}$ -GFP NAAIRS mutant shows inhibited binding to E-cadherin or VE-cadherin. (3A) Immunoblots of co-precipitation experiments examining FF-NAAIRS through KK-NAAIRS $\text{G}\alpha 12^{\text{QL}}$ mutants with the GST-fused 98 C-terminal amino acids of E-cadherin, showing no impaired binding. $\text{G}\alpha 12^{\text{QL}}$ NAAIRS mutants were detected by eGFP antibody. (1B) Densitometric data for the displayed co-precipitation experiments, showing the precipitate to load ratio for each immunoblot.

Previous investigations into vascular endothelial tissue disruption have found that the interaction of $\text{G}\alpha 13$ with VE-cadherin leads to vascular barrier leakiness and inflammation in human lung tissue.⁸ However, this study did not examine $\text{G}\alpha 12$'s ability to bind VE-cadherin. Also, because $\text{G}\alpha 12$ and $\text{G}\alpha 13$ display differing effector interaction, it was sought to determine if preferential binding occurs between the two G proteins and the cadherins tested. Interestingly, it was discovered that the $\text{G}\alpha 12^{\text{QL}}$ bound more strongly to E-cadherin, VE-cadherin, N-cadherin and cadherin-14 compared to $\text{G}\alpha 13^{\text{QL}}$ (Fig. 4A-D).

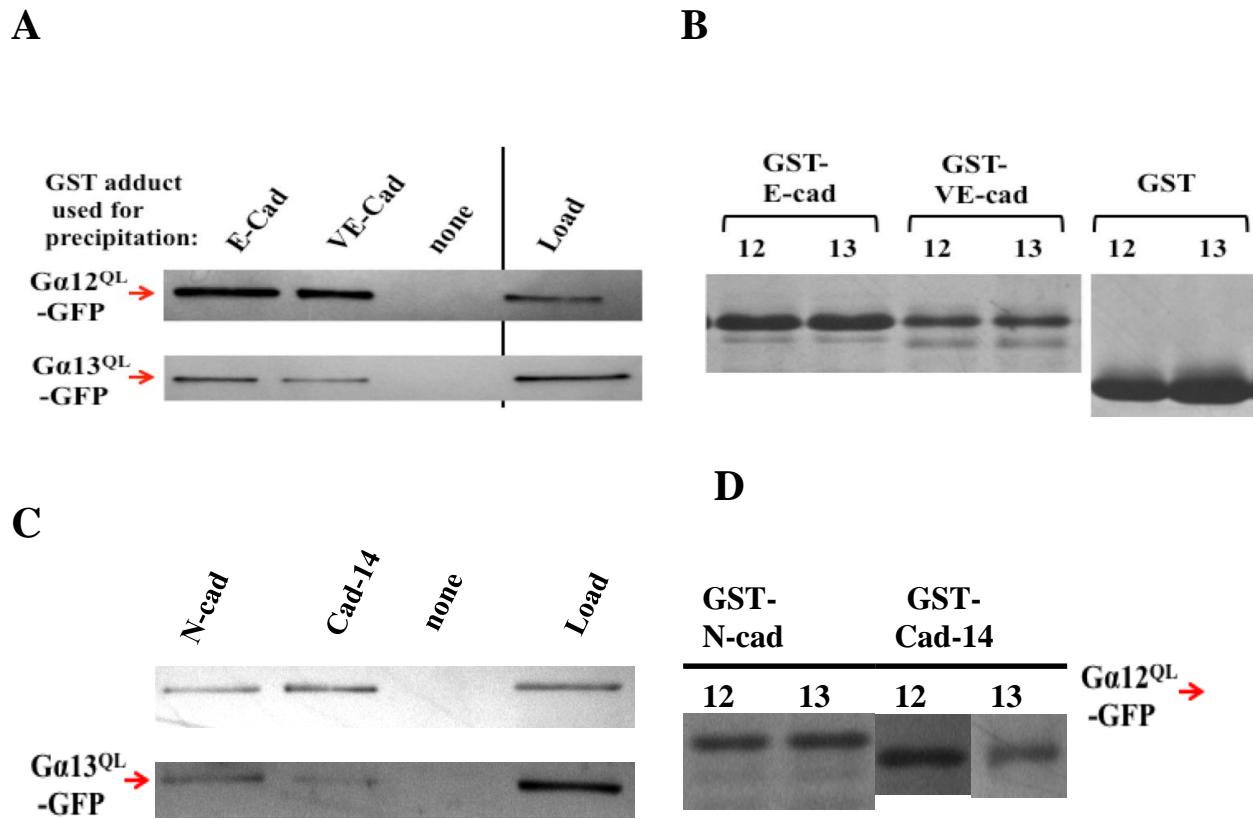


Figure 4. Co-precipitation of activated $\text{G}\alpha 12$ shows preferential binding to all cadherins tested. (1A) Western blot band intensity and precipitate to load ratio shows clear higher affinity binding of $\text{G}\alpha 12^{\text{QL}}$ to E-cadherin and VE-cadherin compared to $\text{G}\alpha 13^{\text{QL}}$. (1B) The Coomassie Blue protein stain is used as a loading control to determine that each experimental co-precipitation received the same amount of available GST-fused cadherin that would be available to bind. This stain reveals that each condition had equal amounts of E-cadherin and VE-cadherin available for interaction. (1C) shows the co-precipitation data for $\text{G}\alpha 12^{\text{QL}}$ and $\text{G}\alpha 13^{\text{QL}}$ binding to N-cadherin and Cadherin-14, with $\text{G}\alpha 12^{\text{QL}}$ exhibiting higher affinity binding. 1D) Coobule stain again showing levels of available GST-fused protein for G protein binding.

To determine which regions of $\text{G}\alpha 12^{\text{QL}}$ and $\text{G}\alpha 13^{\text{QL}}$ were responsible for binding to the cadherins, four chimeric constructs previously described¹⁰ were used in co-precipitation experiments (Fig. 5). Cell lysates expressing the chimeric constructs showed remarkably similar precipitate to load ratios of binding for all the C-terminal tails of the cadherins examined, with cadherin-14, the only type II cadherin tested, showing the most decreased signal. E-cadherin,

VE-cadherin and N-cadherin each showed similarly strong binding affinity to all four chimeras, which, in combination with the uniformly weak cadherin-14 signal, made interpretation of these results particularly difficult (Fig.6).

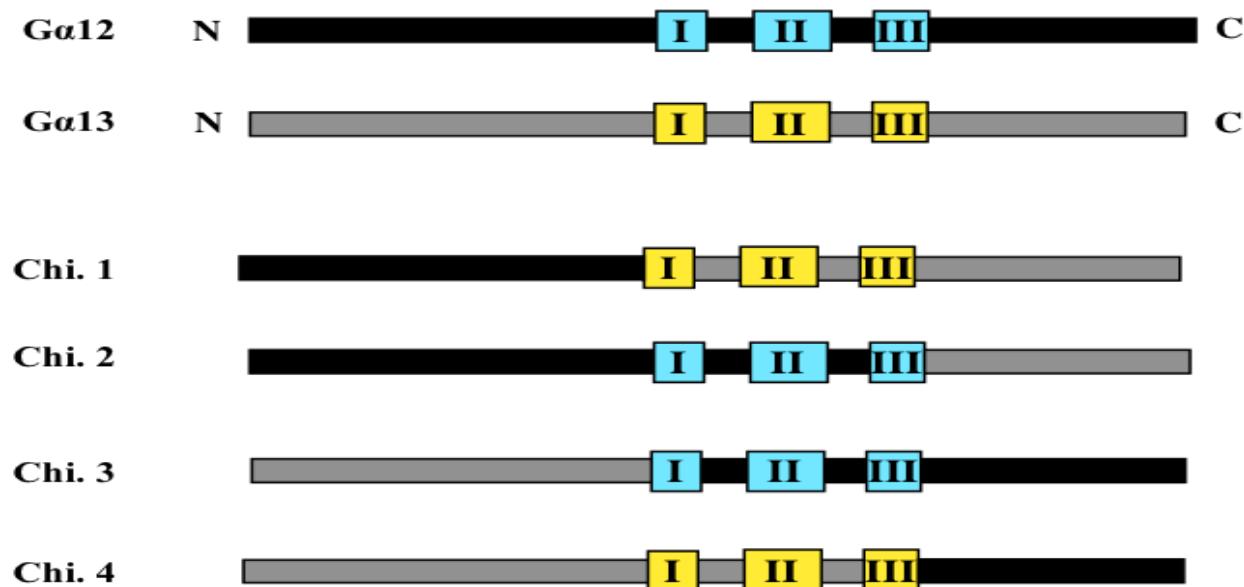


Figure 5. Schematic maps of Gα12, Gα13 and the four chimeras used for co-precipitation with cadherins. Gα12 amino acid sequence is in black with cyan switch regions labeled I, II, and III, while Gα13 sequence is gray, with switch regions highlighted in yellow. Each chimera harbored the constitutively activating Q²²⁹L mutation and was epitope tagged with eGFP.

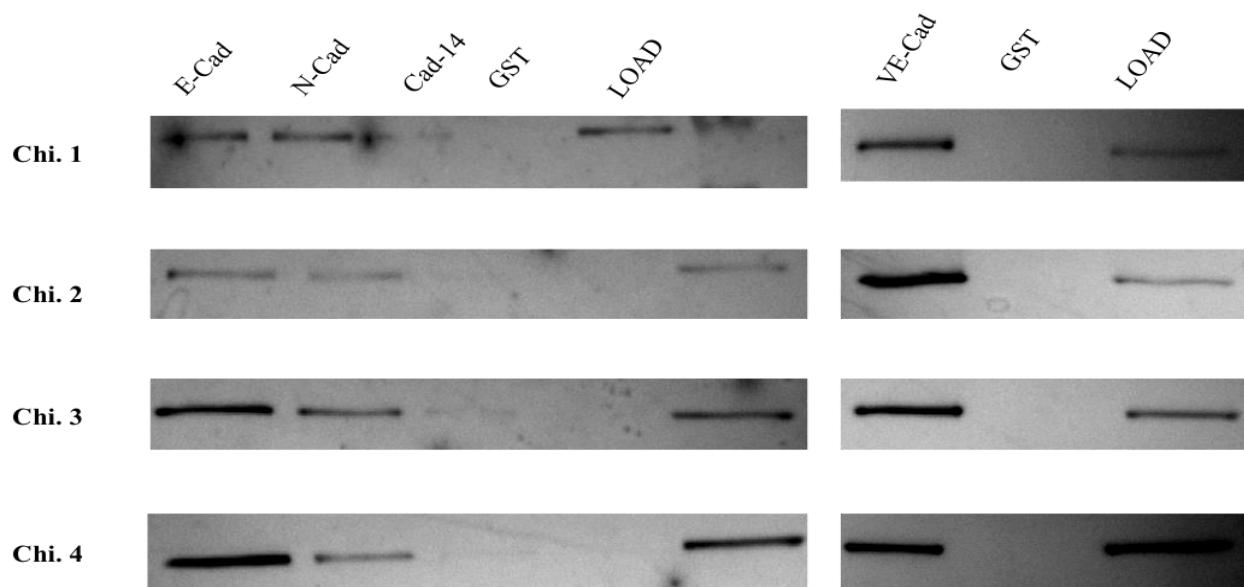


Figure 6. Chimeras of Gα12 and Gα13 fail to delineate specific determinants of binding to cadherins. Representative Western blots of co-precipitation with GST-fused cadherin tail experiments. The cadherins aligned at the top were GST fused and served as the co-precipitation, or “pulldown” agent. The “loads” are at the far right after chimera 1. Each chimera produced similar precipitate to load ratios with every cadherin, except chimera 2 with

VE-cadherin, which showed a strong pulldown signal when compared to the other cadherins precipitated with chimera 2. Protein bands were detected by eGFP antibody as described in section 2.5.

To further investigate previous findings that an acidic 11 amino acid region of the E-cadherin tail is necessary for its interaction of $\text{G}\alpha 12^2$ (Fig. 7A), constitutively active $\text{G}\alpha 13^{\text{QL}}$ was co-precipitated with this E-cadherin deletion mutant. Strikingly, it was found that $\text{G}\alpha 12^{\text{QL}}$ still bound strongly to this deletion mutant (E-cad Δ G12) while $\text{G}\alpha 13^{\text{QL}}$ binding was drastically reduced (Fig. 7B). While surprising, this finding suggests that this region of E-cadherin is also necessary for $\text{G}\alpha 13$ interaction, while also introducing the possibility that these mutationally active constructs may differ in their three dimensional active conformations.

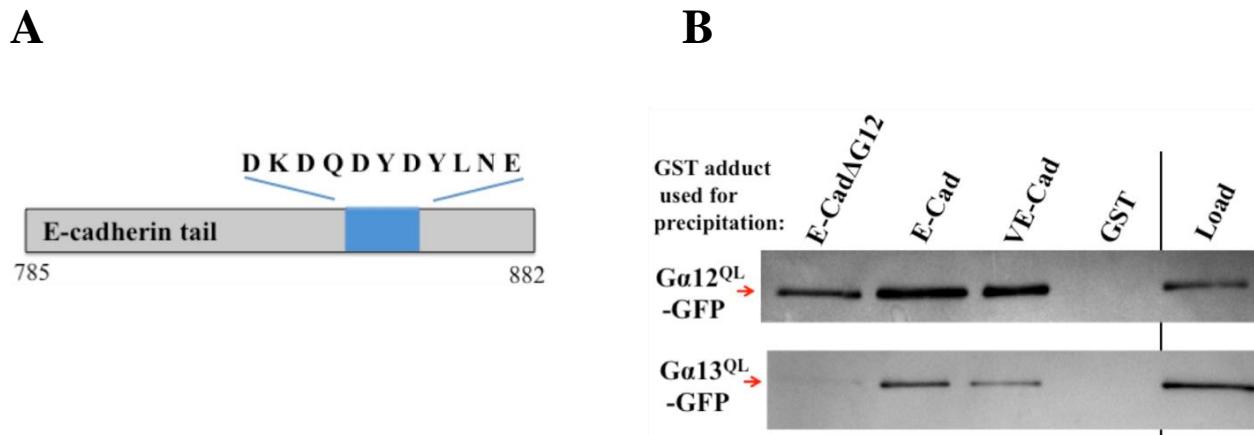


Figure 7. The $\text{G}\alpha 12$ interacting domain of E-cadherin is bound by $\text{G}\alpha 13$. (1A) A schematic map of the 98 C-terminal amino acids of the cytoplasmic tail of E-cadherin. The blue box represents the 11 amino acid region that is deleted in E-cad Δ G12 with its sequence shown in single letter amino acid code. (1B) Western blots showing the abrogated binding of $\text{G}\alpha 13^{\text{QL}}$ -GFP to the E-cad Δ G12 mutant in parallel with E-cadherin and VE-cadherin, while again demonstrating the preferential binding of $\text{G}\alpha 12$ to E-cadherin and VE-cadherin. Immunoblots shown are representative of four separate trials.

4. Discussion

Present results have failed to reveal which residues of $\text{G}\alpha 12$ or $\text{G}\alpha 13$ are responsible for their interaction with cadherin cell adhesion molecules. The failure of the $\text{G}\alpha 12^{\text{QL}}$ NAAIRS mutants of the switch region I to uncouple interaction with both E-cadherin and VE-cadherin was surprising, especially when compared to previous investigations into the interaction of $\text{G}\alpha 13$ with VE-cadherin, which found this region necessary for the interaction. Still, the possibility remains that although these G proteins share a high level of homology, this region of $\text{G}\alpha 12$ may not be involved with its interaction with cadherin proteins. Interestingly though, two switch region I NAAIRS mutants, HH and II have proved successful in uncoupling interaction with another class of cell adhesion protein, $\beta 3$ integrin.¹¹ A potentially useful tool for future experimentation could involve the production of these NAAIRS mutants in $\text{G}\alpha 13$, which could be utilized in similar co-precipitation assays to replicate previous research into its interaction with VE-cadherin, and to further test the other cadherins studied here. Constitutively active $\text{G}\alpha 12$ consistently showed higher affinity binding to all the cadherin molecules tested. This finding could suggest that $\text{G}\alpha 12$ may play a larger physiological and pathological role in the regulation of these adhesion proteins than $\text{G}\alpha 13$, though both $\text{G}\alpha$ subunits can bind these molecules, and have been shown to disrupt cell adhesion.¹² The nebulous results from the chimeric analysis could be explained by the highly similar amino acid structures of both $\text{G}\alpha 12$ and $\text{G}\alpha 13$. Both $\text{G}\alpha 12$ and $\text{G}\alpha 13$ effectively bind to the cadherins examined, and it could be that the residues necessary for the interaction exist in both proteins and are still functional in the four chimeras. More specific chimeric constructs that swap switch region I of both $\text{G}\alpha$ subunits could potentially illuminate the determinants of binding to these adhesion proteins. Though the residues of $\text{G}\alpha 12$ and $\text{G}\alpha 13$ responsible for binding cadherins were not determined, it was found that $\text{G}\alpha 13$ binds the same 11 amino acid region of the E-cadherin cytoplasmic tail. More specific mutagenesis of this 11 amino acid region of E-cadherin

could reveal the individual residues that mediate this interaction with both G α subunits. The seemingly contradictory result that activated G α 12 still binds this E-cadherin deletion mutant could be explained by the activation strategy used. This study was performed using mutationally activated G α 12^{QL}, in which glutamine²²⁹ is mutated to a lysine residue. This mutation creates a GTPase deficient protein, simulating the active conformation. The previous finding that G α 12 cannot bind this E-cadherin deletion mutant used purified G α 12 that was activated by GTP γ S loading.² It is possible that these two different activation methods could create different activational conformations of the protein. Furthermore, these QL mutationally active constructs are derived from whole cell lysates of HEK293 cells; this could also contribute to differing results due to the cellular context in which these constructs are produced. Another activational mutant of G α 12, arginine²⁰³ to cysteine has been engineered by the author and is currently being tested for binding to this E-cad Δ G12 mutant and for stimulation of SRE response to illuminate possible discrepancies between this mutant and the canonical QL activating form.

5. Acknowledgements

Funding from the GlaxoSmithKline and C.D. Spangler foundations, in addition to the Lineberger Comprehensive Cancer Center, supported this work. The author would like to sincerely thank Dr. Thomas E. Meigs for his indispensible support, mentorship and expertise involved in this project, along with the Undergraduate Research Program of UNCA. Further thanks is extended to previous lab members Janie Warstler and Kayla Huffman for their past work involving cell adhesion proteins.

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