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Selective Interaction of β3 Integrin with Guanine Nucleotide-Binding Proteins Gα12 and Gα13

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Abstract

Cell attachment, migration, and proliferation are essential processes for both normal tissue growth and malignant development of cancers. Guanine nucleotide-binding (G) proteins and cell surface proteins of the integrin family play key roles in the regulation of these processes. The G proteins Gα12 and Gα13 have 66% amino acid homology, yet differ in their downstream signaling function. While these proteins have been extensively studied in their own roles, the mechanisms by which they interact have only been partially examined. Other investigators have shown C-terminal binding of β3 integrin to Gα13, but not Gα12. In the Meigs lab, G protein affinity for β3 integrin was examined utilizing epitope-tagged forms of Gα12 and Gα13. It was found that Gα12 showed greater binding affinity for β3 integrin than did Gα13, in both myc- and GFP-tagged forms. Furthermore, constitutively active and inactive forms of Gα12 and Gα13, as well as wild-type Gα12/13, were utilized to determine if this differential was affected by the activation state. For Gα12, β3 integrin exhibited stronger binding for the GTP-bound form than the wild-type or GDP-bound forms. These findings suggest that Gα12 must be in the GTP-bound form to adequately bind β3 integrin in cells. In regards to Gα13, β3 showed strongly impaired binding in all activation states. Lastly, NAAIRS sextet mutations were tested to attain greater knowledge of specific structural features of Gα12 required for interaction with β3 integrin[1]. Unexpectedly, two sextet amino acid substitution mutants of Gα12 exhibited impaired binding to β3 integrin. This provides new structural details and serves as a basis for further research in examining these regions to specifically identify the properties that disallow binding action. As both Gα12 and Gα13 have been implicated in cancer pathways, understanding these interactions is critical for complete mechanistic understanding of the metastatic potential of epithelial cancer cells.

1. Introduction

Heterotrimeric guanine nucleotide-binding (G) proteins play essential roles in cancer progression, metastatic invasion, and embryonic development. Better understanding of the cellular signaling processes that allow these to occur is integral for the future of potential antineoplastic drugs. These proteins consist of α, β, and γ subunits, which rely on GDP-GTP exchange for activation[2]. Upon activation, the α subunit dissociates with the β/γ subunits and serves as the major signaling molecule for its downstream effectors[3]. Gα12 and Gα13 are members of the G12/13 class of these proteins. While these proteins have similar sequence homology, they invoke distinct downstream signaling processes. Gα13 has been shown to interact with and regulate the function of β3 integrin, but there is little data on the Gα12/β3 integrin interaction published[4].

Integrins are heterodimeric integral transmembrane protein receptors consisting of α and β subunits, with a large extracellular domain and a small cytoplasmic tail. They are heavily involved in cell surface-mediated signaling, specifically between the actin skeleton and extracellular matrix during cell migration. Likewise, they play a critical role in thrombosis and hemostasis, which involves outside-in signaling related to platelet spreading[5]. Integrins

participate in two different types of signaling: outside-in and inside-out. This inside-out signaling activates ligand binding, and cellular stimulation triggers clustering or altered conformation of the integrin subunits to increase their affinity for ligands[4]. Outside-in signaling then mediates the responses induced by the ligand binding, and "signals are transduced to the cytoplasm, which induces a cascade of intracellular signaling events, including protein phosphorylation and cytoskeletal reorganization"[6]. Downstream effects of this signaling also include spreading, retraction, migration and proliferation.

The impetus for this research began with findings that outside-in and inside-out signaling both require G protein interaction, and that crosstalk occurs between G-protein coupled receptors (GPCR) and β3 integrin pathways[2]. Furthermore, Gong *et al.* elucidated that Gα13-integrin interaction was supported in the GTP-bound, constitutively active form of Gα13, through binding at the cytoplasmic domain of β3 integrin[7]. Given that cadherins have been shown to interact robustly with both G protein families, the commonalities of cadherins and integrins in the Gα12/13 domains used for binding were examined. A comparative binding study of Gα12 versus Gα13 was performed, and the structural determinants that evolved to allow binding and regulation of β3 integrin were examined. Furthermore, it was sought to determine whether the activation state of the G proteins had any effect on binding affinity. Cassette mutational analysis was employed to identify the regions of Gα12 necessary for β3 integrin binding, as well as epitope-tagged variants of Gα12 and Gα13.

2. Materials and Methods

2.1 Creation of GST β3 Integrin Fusion

Based on the recent finding of Gong *et al.* that the cytoplasmic domain of β3 integrin interacts with Gα13, this domain was examined for interaction with Gα12 in comparison to Gα13[4]. GST-fusions were created by polymerase chain reaction (PCR)-amplifying the amino acids comprising this cytoplasmic tail of β3-integrin from human and mouse brain cDNA libraries. Published sequences for human (GenBank: AGC54784.1) and mouse (AAB94086.1) B3 integrin were examined and oligonucleotides were selected to hybridize to amino acid sequences 742-788 and 741-787, respectively. Flanking this amplicon, BamHI (upstream, 5'-GGATCC-3') and SalI (downstream, 5'-CAGCTG-3') restriction endonuclease sites were engineered to allow ligation into pGEX-5X-1 plasmid vector (GE Healthcare). Two arbitrary nucleotides were added in the forward primer between the BamHI and B3 integrin start codon site in order to preserve the correct reading frame for the protein. Likewise, a stop codon was introduced in the reverse primer. The PCR oligonucleotides used (Eurofins Genomics) are as follows:

Human: 5' - CAG GGA TCC CTA AAC TCC TCA TCA CCA TCC AC -3'
3' -GT TAT AGT GCA TGG CCC CGT GAA CTC AGC TGG ACC -5'

Mouse: 5' - CAG GGA TCC AAG CTA CTC ATC ACC ATT CAT G -3'
3' - G TTA TAG TGG ATG GCC CCC TGA ACT CTT AAG GCC-5'

2.1.1 Polymerase Chain Reaction to Probe cDNA Libraries with β3 Integrin Oligonucleotides

Two unique standard PCR solutions were created for the human and mouse samples. Each consisted of 15μL 10x PCR buffer, 4.5μL dNTPs, 15μL FW human (or mouse) diluted stock oligonucleotide, 15μL RV human (or mouse) diluted stock oligonucleotide, 7.5μL DMSO, 1.5μL Pfu Cx Turbo polymerase (Agilent Technologies), and 88.5 μL ddH_2O. Each standard solution was separated into three PCR tubes, at 49μL per tube, plus 1μL of the isolated epithelial DNA or cDNA of interest. The final contents of each tube that underwent PCR are shown in Table 2. The samples underwent PCR conditions at 95°C and 72°C, for a total of 30 cycles, and were subjected to a final hold temperature of 4°C. The contents of these tubes were then dyed for visualization and run on a 1.5% (w/v) agarose gel. The results of this gel are shown in Figure 1. These constructs were also verified by sequencing.

Table 2. PCR hybridization tube contents. Each tube contains 49µL PCR solution, plus the reagents noted below.

	1	2	3
Human	1µL human epithelial cheek cell (personal) DNA	1µL 0.382H cDNA	1µL human brain cDNA
Mouse	1µL mouse brain cDNA	1µL 9.5 day mouse cDNA	1µL 10.5 day mouse cDNA

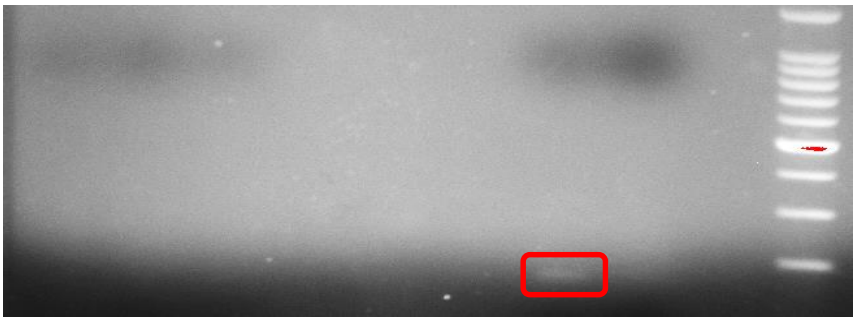

Figure 1. Gel electrophoresis results for presence of β3 integrin encoding sequence in various human and mouse cDNA samples. The only sample that effectively hybridized to the PCR oligonucleotides was the human brain cDNA (0.328H). The hybridized 0.328H cDNA was then PCR-cleaned using a Promega Wizard® SV Gel and PCR Clean-Up System following the protocol of the manufacturer.

2.2 Expression and Purification of GST β3 Integrin Fusion Protein

Plasmid vectors pGEX-5X-1 harboring the cytoplasmic region of β3 integrin were transformed into BL21-Gold (DE3) *E. Coli* cells for the GST protein prep. 12 ml cultures of Luria-Bertani (LB) broth containing ampicillin (Amp) at a concentration of 75 µg/ml were inoculated with singular colonies of the GST β3 integrin construct. These cultures were allowed to shake at 220 rpm at 37C for 12-16 hours. 5mL of this culture was then added to a 500 mL LB-ampicillin culture at the same ampicillin concentration, and was 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 the culture. This mixture was then allowed to incubate on the shaker at 220rpm, 37°C for an additional 2.5 to 3 hours. Once evenly divided into three separate bottles, the cultures were centrifuged at 4°C for 15 minutes at 6000rpm. The supernatants were discarded and the bacterial pellets were placed on ice. One 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 the second and third pellets to homogenize them as well. 4-5 mg lysozyme (MP Biomedicals) was added to the 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 tube was rocked every two minutes by hand until viscosity significantly decreased. It was 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 supernatant of the bacterial tube was then split evenly among the two 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 snap frozen in liquid nitrogen, and stored at -80C.

2.4 Co-precipitation Experiments with β3 Integrin and Gα12/13

To detect interaction between GST β3 integrin lysates and Gα12, Gα13, and their mutants, coprecipitation assays were performed. The G protein lysates were mixed with HEDM 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 β3 integrin fusion lysates also were suspended in $H_{50}E_1D_3M_{10}$ (50mM Hepes, 1mM EDTA, 3mM DTT, 10mM $MgSO_4$) 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 (see Figure 2). Immunoblot quantitative data was obtained using Kodak GelLogic 100 imaging system and Carestream Molecular Imaging 5.X software to calculate Gaussian fit.

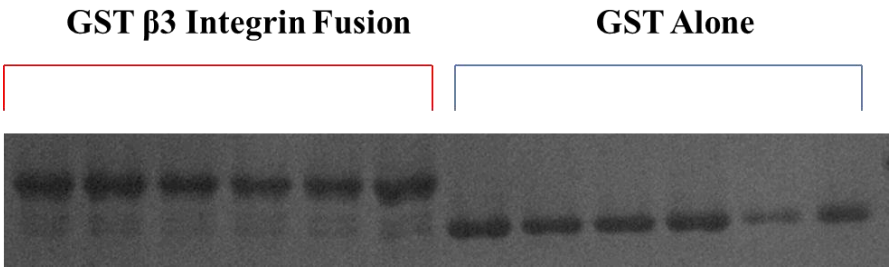

Figure 2. Coomassie Blue stain of GST β3 integrin fusion samples confirming integration of the β3 integrin coding sequence into the GST-expressing plasmid.

3. Results

To determine whether β3 integrin exhibits binding to constitutively active (GTP-bound) Gα12 and Gα13, co-precipitation assays were performed (see Fig. 3). These GTP-bound forms were obtained using a mutational strategy involving a glutamine to leucine (Q229L) switch at amino acid 229 (Q229L). We utilized Gα12- and Gα13-specific primary antibodies to detect binding of the myc-tagged G proteins in our initial experiments, and for our studies directly comparing Gα12 and Gα13 affinity for β3 integrin, GFP-tagged variants of these G proteins were utilized and detected them with an anti-GFP antibody. Surprisingly, that Gα13 exhibited impaired binding affinity with both myc- and GFP-tags, while Gα12 exhibited robust binding in both tagged forms.

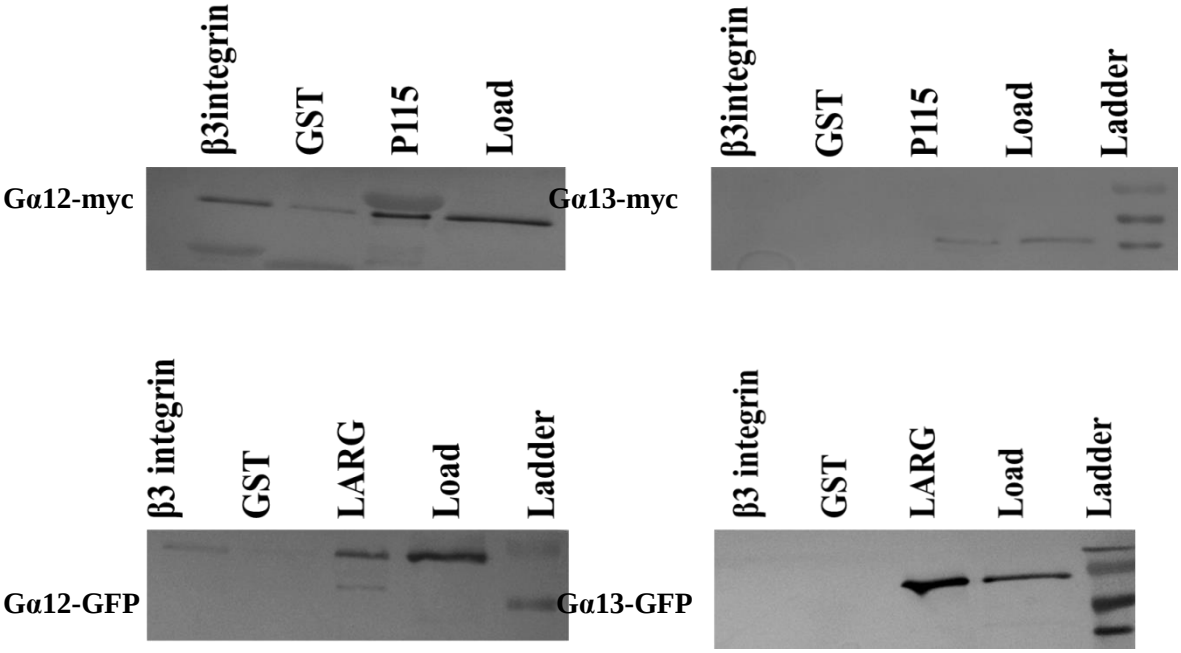

Figure 3. $G\alpha12^{QL}$, but not $G\alpha13^{QL}$, interacts with β3 integrin. The "load" samples serve as a measure of comparative protein abundance, p115 and LARG serve as positive controls, and GST serves as a negative control for β3 integrin/Gα12 pulldown. Immunoblots shown are representative of 3 independent experiments.

To further examine the specific structural determinants within Gα12 that mediate β3 integrin binding, NAAIRS cassette substitution mutants were used. To determine the regions within Gα12 specifically responsible for binding affinity of β3 integrin, a series of well-tolerated amino acids, asn-ala-ala-ile-arg-ser (NAAIRS), were substituted into various sextuplets of myc-tagged $G\alpha12^{QL}$. All mutants were constructed in constitutively active (Q229L) Gα12. The alphabetical designations of these NAAIRS mutants are depicted in Figure 4, with the Switch I region highlighted in gray[1].

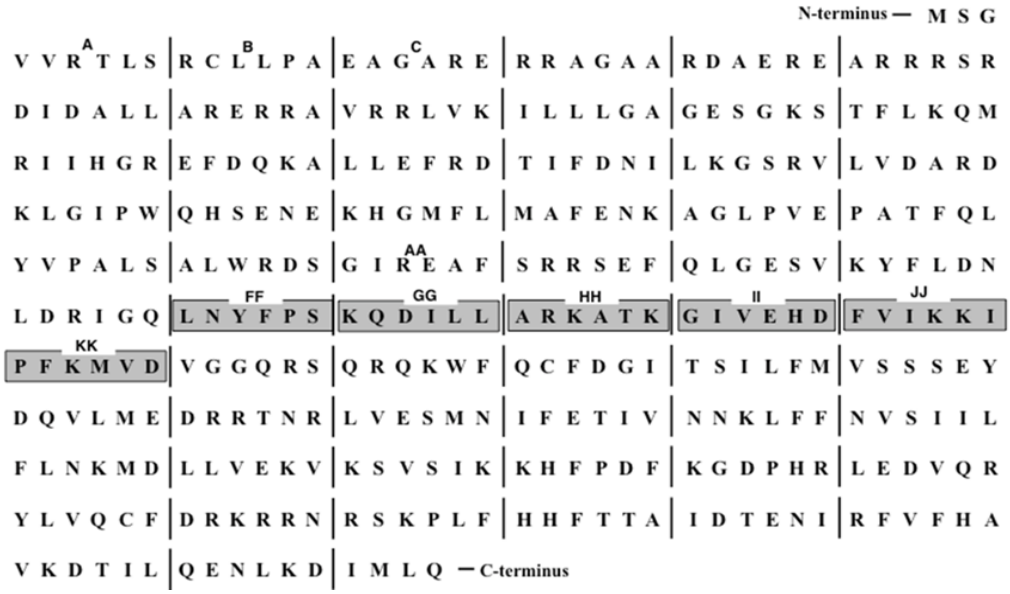

Figure 4. NAAIRS cassette substitution mutants for use in co-precipitation assays.

Since the Switch I region had previously been implicated in binding with cadherins, another class of related cell surface adhesion proteins[7], NAAIRS mutants spanning this region were tested for similar binding in β3 integrin. Gα12^{QL}-myc was used as a positive control, and a blank lysate as a negative control (see Fig. 5). NAAIRS mutant II showed impaired binding to β3 integrin as compared with the positive control, and mutant HH showed complete disruption of binding.

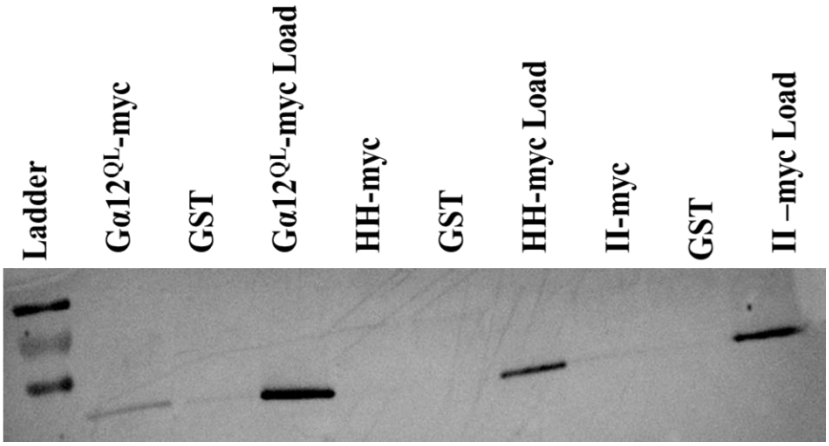

Figure 5. Identification of determinants within Gα12 that mediate β3 integrin binding. Immunoblots shown are representative of 2 independent experiments.

To determine whether interaction of β3 integrin with Gα12/13 was reliant on a specific activation state, compared constitutively active Gα12^{QL}-myc was compared with Gα12 wild-type and constitutively inactive (GDP-bound) G228A in co-precipitation experiments. Gα12^{QL}-myc showed greater binding affinity than Gα12 wild-type or G228A. Gα13 did not display any detectable binding, regardless of activation state (see Fig. 6a). Quantification of binding data was also performed for these two co-precipitation assays. Gaussian fit values were determined for bands on immunoblots and used to calculate precipitate-to-load densitometric ratios (Fig. 6b).

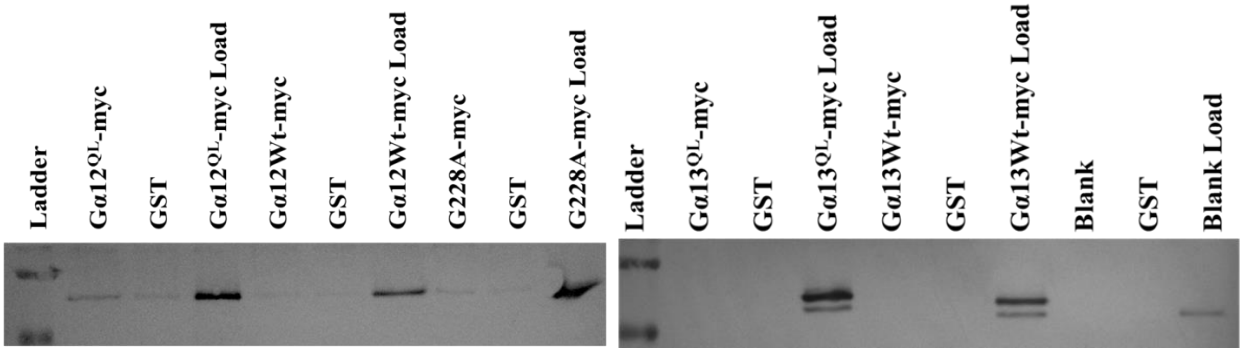

Figure 6a. Activation dependence for β3 Integrin interaction. Immunoblots shown are representative of 3 independent experiments.

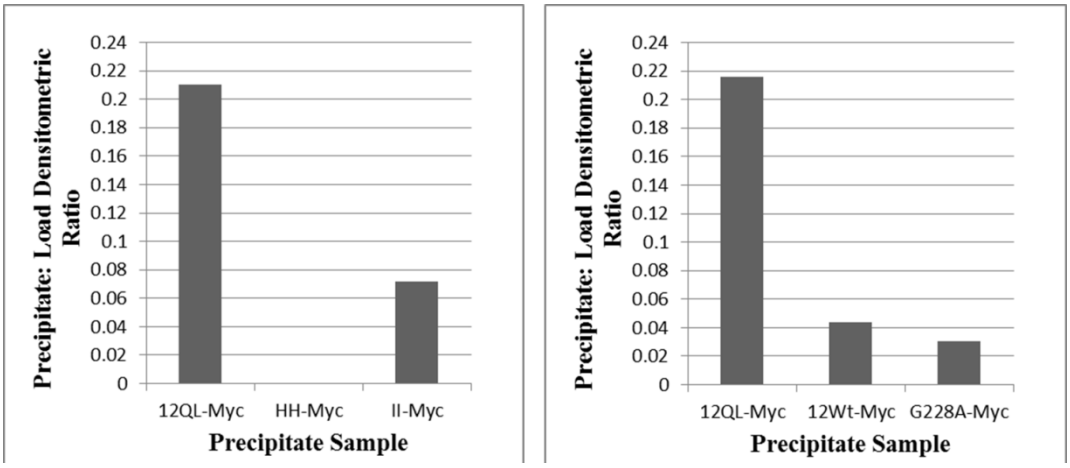

Figure 6b. Precipitate-to-Load Densitometric Data

4. Discussion

Unexpectedly, myc-tagged Gα12, but not myc-tagged Gα13, interacted with β3 integrin. This was similarly demonstrated in that Gα12-GFP interacted with β3 integrin whereas Gα13-GFP did not. Therefore, Gα13 exhibits impaired binding affinity with both myc- and GFP-tags, suggesting that it is not a function of epitope tag blocking binding but rather that the protein as a whole does not interact with β3 integrin in the constitutively active state. This implicates data never before elucidated about the binding capabilities of β3 integrin, and suggests that Gα12 plays a greater role in β3 integrin interaction than does Gα13. This could be especially significant not only due to the potential for the crosstalk of downstream pathways, but for the direct physical binding effects that may arise from this interaction. As has been previously shown, when Gα13 is bound to β3 integrin, c-Src is active, RhoA is inhibited, and platelet spreading occurs[4]. This implies that the basic, physical interaction of the G protein with β3 integrin bolsters the natural activity of integrins, which may be clinically applicable in a medical setting. Further elucidation of how this physical binding affects either β3 integrin or Gα12 is needed, and could be completed by testing known binding targets of β3 integrin such as Talin, to see if activity is inhibited or stimulated[8].

Co-precipitation assays with Switch I region NAAIRS mutants displayed similarly interesting data regarding the uniqueness of β3 integrin interaction with G proteins. The impairment seen in NAAIRS region II and complete blockage in region HH is interesting in comparison to binding exhibited by cadherins. Previous data suggests these regions (within Switch I) of Ga13 are potentially necessary for binding to vascular endothelial (VE) cadherin[7]. Thus, we may conclude that the Switch I regions in both Gα12 and Gα13 evolutionarily represent integral and relatively functionally unchanged domains, as they both seem to be necessary for the binding of these cell surface adhesion proteins.

Upon performing activation state co-precipitation assays, it became apparent that activational state had little effect on the binding capabilities of β3 integrin and Gα12/13 proteins. This serves as further evidence that the results achieved describing the impaired binding of Gα13 with β3 integrin is generalized. The disparity in the data between our lab and that of previous researchers may have to do with the activational mutagenesis strategy used. In the Meigs lab, a Q229L point mutation strategy in Gα12/13 was employed, whereas others have used a truncation strategy to test interaction[4].

Further directions for this research include a further comparative pulldown of Gα13 including G225A (constitutively inactive), to determine if an inactive (GDP-bound) form is capable of interaction with β3 integrin. To better elucidate which structural determinants of Gα12 confer binding specificity with β3 integrin, further studies with NAAIRS mutant co-precipitation including within and flanking the Switch I Region may be completed. Additionally, the use of Gα12/Gα13 chimeras may reveal the structural basis of preferential Gα12 binding to adhesion proteins. Lastly, studies of the cell biological role of Gα12-β3 integrin interaction using selectively uncoupled Gα12 mutants and inhibitory peptides may also confer novel data about β3 integrin and what physiological implications it may have.

6. Acknowledgements

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7. Resources

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