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Gα12 and Gα13 disrupt PTP1B binding to the cadherin-catenin complex

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Abstract

Cadherins are membrane spanning proteins that play an important role in cellular adhesion. The extracellular region of E-cadherin adheres to other nearby cadherins through homotypic binding, holding cells together, while its cytoplasmic tail is anchored to the actin cytoskeleton with a complex of other proteins including beta-catenin. When Tyr-654 on beta-catenin is phosphorylated, it shows less affinity for E-cadherin binding and cell-cell adhesion is compromised. Previous work has shown that the protein tyrosine phosphatase, PTP1B, continuously dephosphorylates beta-catenin at the Tyr-654 residue, however more data has been gathered on PTP1B's interactions with N-cadherin than E-cadherin. PTP1B and its interaction with beta-catenin is important to N-cadherin being properly trafficked from the ER to the cell membrane. The G12/13 subfamily of heterotrimeric guanine nucleotide binding proteins (G-proteins) have also been shown to interact with cadherins and cause decreased cell adhesion function. Gα12 and Gα13 both bind to a region on N-cadherin that overlaps with the PTP1B binding region. This region is highly preserved in E-cadherin, so it is predicted that PTP1B also binds with E-cadherin. Because the G12/13 class of G-proteins and PTP1B bind the cadherin cytoplasmic domain at overlapping positions, a hypothesis emerges that Gα12/13 displaces PTP1B from cadherins. It would be expected that without the binding of PTP1B, beta-catenin will remain in a phosphorylated state that hinders its binding to cadherins. To test this a myc-tagged PTP1B protein was engineered and transfected into cultured cells for protein interaction studies. Through pulldown analysis and immunoblotting it has been shown that PTP1B and E-cadherin do interact. Work is on going to determine if Gα12/13 signaling has an effect on PTP1B binding. Further experiments will examine beta-catenin to determine if Gα12/13 signaling effects on PTP1B also affect beta-catenin binding to E-cadherin.

1. Introduction

Cadherins are a family of integral membrane proteins that contribute to cell adhesion through calcium-dependent binding. Classical cadherin structure consists of an extracellular region of tandem repeats, a transmembrane domain, and a cytoplasmic tail that is essential for anchoring the cadherin into the actin cytoskeleton.[1] The extracellular region will bind with neighboring extracellular cadherin domains through homotypic binding for cellular adhesion. Epithelial cells will typically express E-cadherin, while N-cadherin is expressed in neuronal adherens junctions.[2,3] In addition, N-cadherin is known to be essential for normal embryonic and heart development while E-cadherin is essential for normal epithelial cell morphology.[4] Many epithelial cancer cells undergo cadherin switching from E-cadherin to N-cadherin, which has been shown to increase motility and cell proliferation into other tissues.[2]

Binding to the cytoplasmic domain of cadherins is a network of proteins that contribute to anchoring them to the actin cytoskeleton (Figure 1). Beta-catenin is important for cellular adhesion function, acting as a bridge between the cadherin and alpha-catenin, which binds directly to actin filaments in the cytoskeleton.[4,5] Phosphorylation of specific tyrosine residues within the beta-catenin sequence will undo this complex. Phosphorylation of Tyr-654 will cause a loss of beta-catenin binding to the cadherin and phosphorylation of Tyr-142 will cause loss of binding between beta-catenin and alpha-catenin.[5] The protein tyrosine phosphatase, PTP1B, works to continuously dephosphorylate beta-

catenin at the Tyr-654 residue to maintain cadherin binding. PTP1B binds to the N-terminus of N-cadherin following the region essential for beta-catenin binding.[6] Because the region on the N-cadherin cytoplasmic tail is highly preserved in E-cadherin (Figure 2), this suggests that PTP1B will also bind to E-cadherin in the same region. PTP1B binding is also essential for N-cadherin trafficking from the endoplasmic reticulum to the cell membrane. After the mature cadherin is synthesized in the ER, beta-catenin and p-120 catenin must bind to the cadherin in order for it to be recognized for trafficking up to the cell membrane.[7] PTP1B must be present and available to dephosphorylate beta-catenin in order for binding to occur.[7] This same mechanism has also been observed in cells that express E-cadherin.[7]

Among the kinases that will phosphorylate beta-catenin, the Src Family kinases target Tyr-654 on beta-catenin as well as targeting N-cadherin for phosphorylation.[5,8] PTP1B, when bound to the cadherin, will dephosphorylate beta-catenin at Tyr-654 and prevent dissociation from the cadherin. It has also been shown that activation of Src will lead to phosphorylation of N-cadherin and the loss of beta-catenin binding during the transendothelial migration of cancer cells.[8]

Heterotrimeric guanine nucleotide binding proteins (G-proteins) have many known functions in cellular signaling. The $G_{12/13}$ subfamily of G-proteins has previously been shown to cause the release of beta-catenin upon binding to the cytoplasmic tail of cadherins.[9,10] There is a region of E-cadherin that is essential for Gα12 and Gα13 binding.[11] This region shares an overlapping sequence with the binding region for PTP1B on the cadherin.

It can be hypothesized that because PTP1B and Gα12/13 share an overlapping binding zone that the proteins have the potential to be competitive inhibitors to one another. Therefore, a possible mechanism for the loss of E-cadherin function can be outlined. If Gα12/13 binds to E-cadherin in place of PTP1B, beta-catenin is no longer dephosphorylated by PTP1B, giving room for Src to phosphorylate beta-catenin (Figure 3). In its phosphorylated state, beta-catenin shows less affinity for E-cadherin and cell adhesion is compromised. In order to test this hypothesis, an epitope tagged PTP1B was created for pulldown analysis and Western blot experiments.

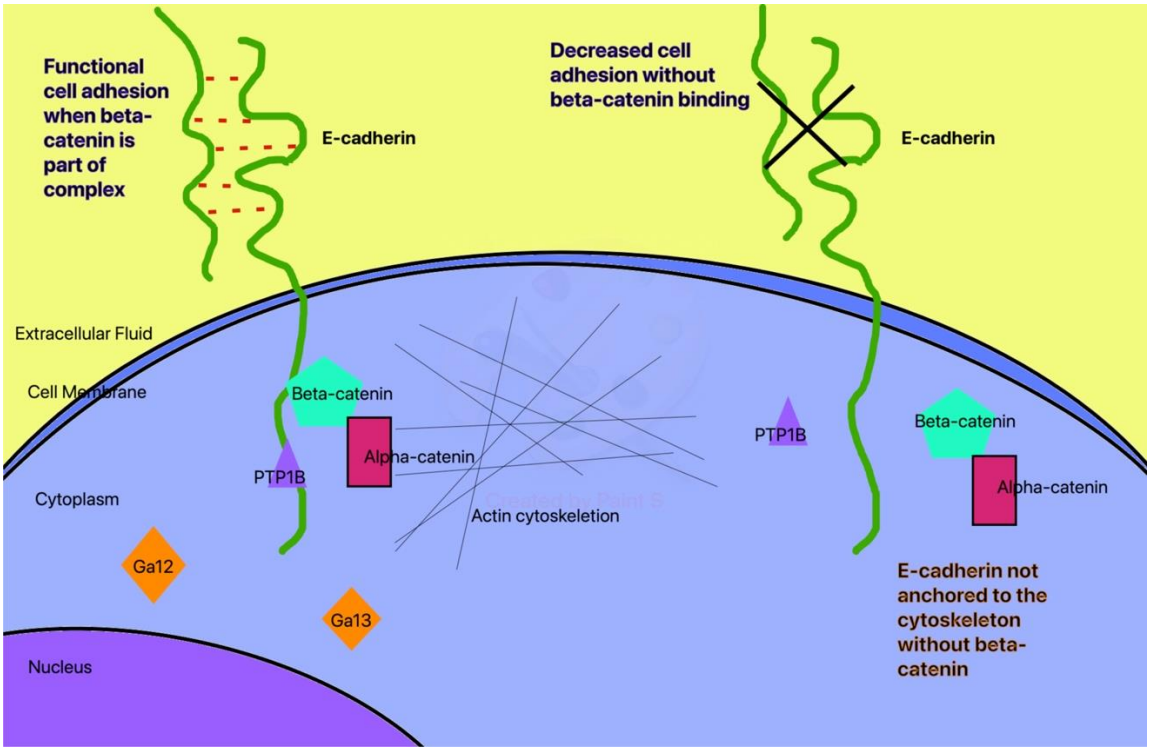


Figure 1. E-cadherin/beta-catenin complex. Beta-catenin binds to the cytoplasmic tail of E-cadherin and to alpha-catenin, which tethers the cadherin to the actin cytoskeleton. Without beta-catenin bound to the cytoplasmic tail, E-cadherin does not function properly for cell-cell adhesion.

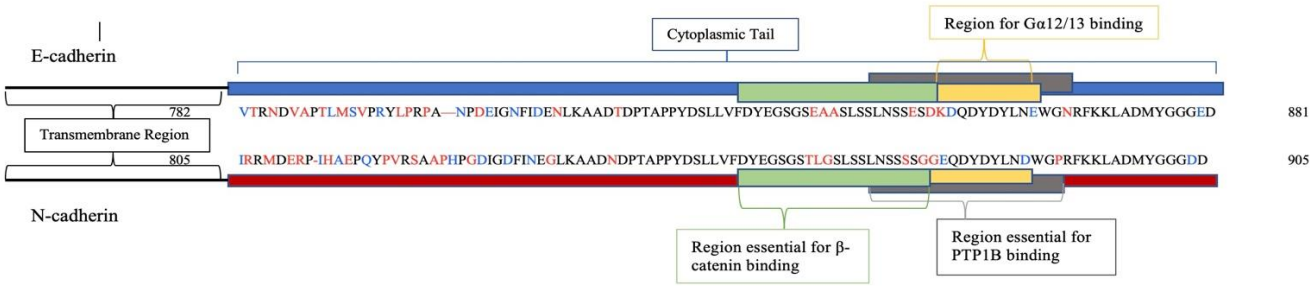


Figure 2. Binding regions on E-cadherin and N-cadherin. Amino acid sequence for N-cadherin (GenBank Accession Number: AAB22854) and E-cadherin (GenBank Accession Number: CAA78353) are shown (black residues are identical, blue residues are similar, red residues show differences). Binding regions for beta-catenin, PTP1B, and Gα12/13 are also shown. Beta-catenin and PTP1B share a short overlapping region. PTP1B and Gα12/13 bind in the same region on cadherins, suggesting competition between the two.

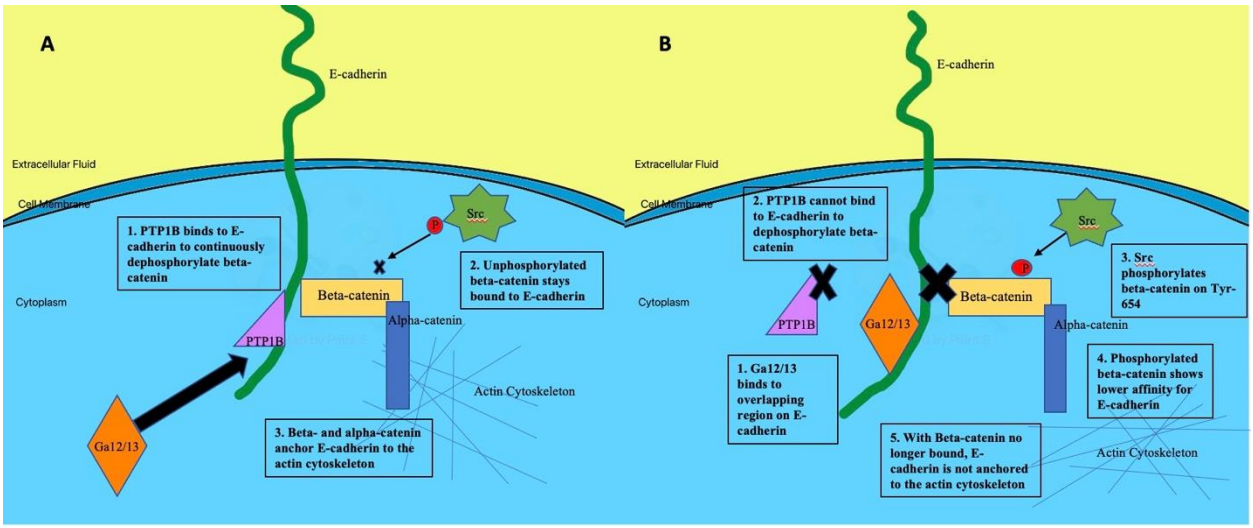


Figure 3. Hypothesized mechanism for reduced beta-catenin binding and loss of cellular adhesion function.

2. Methods and Materials

2.1 Preparation of Epitope tagged PTP1B Protein

A variant of the PTP1B protein was engineered with the addition of a myc-tag to the N-terminus. Oligonucleotides were designed to include restriction digest sequences for EcoRI and Xbal along with the sequence for the myc-tag (EQKLISEEDL) preceded by a Methionine attached to the 5'-end and ordered from Eurofins. Oligos were subjected to PCR with plasmid DNA (pcDNA3.1) containing the sequence for PTP1B. Primary PCR products were then run through agarose gel and the DNA extracted. The final gel-extraction yielded the restriction-digested pcDNA3.1 vector and restriction-digested PCR product. These were joined together via a ligation reaction, which was then transformed into competent JM109 bacteria and plated on LB agar infused with 100 ug/mL ampicillin to select for cells containing re-circularized plasmids. Several resulting colonies were grown under ampicillin selection in LB broth and subjected to plasmid purification (Qiagen) followed by sequencing (GeneWiz).

2.2 Preparation of Cell Lysates for Pulldown Analysis

HEK293 cells were grown in 10-cm dishes with serum-free DMEM and transfected with 10ug of plasmid DNA encoding PTP1B-myc and an activated variant of Gα12 and Gα13 (containing a Q-to-L amino acid substitution that abolishes GTPase activity) using a solution of 2 mg/mL polyethylenimine (PEI). 40-44 hours after transfection, cells were rinsed with cold PBS, scraped and centrifuged at 500 x g for 3 minutes. Pellets were resuspended in Lysis buffer (50mM HEPES pH 7.5, 1mM EDTA, 3mM dithiothreitol, 10mM $MgSO_4$, 1% polyoxyethylene-10-lauryl ether) that was supplemented with the protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1.67 mM), leupeptin (2.1 mM), pepstatin (1.45 mM), Nα-tosyl-L-lysine chloromethyl ketone (58 mM), tosyl-L-phenylalanylchloromethane (61 mM), and phenylmethylsulfonyl fluoride (267 mM). Lysates were continuously inverted for 30 minutes at 4°C and then centrifuged at 80,000 x g for 60 minutes at 4°C. Supernatants were aliquoted and snap frozen with liquid nitrogen and stored at -80°C for future experiments.

2.3 GST Fusion Constructs for E-cadherin and N-cadherin

GST-fusion constructs were transformed into BL21-DE3 cells. Single bacterial colonies were then isolated and used to inoculate 5-mL liquid cultures that contained a 75ug/mL concentration of ampicillin for growth overnight at 37°C. These cultures were transferred into 150-mL cultures with 75ug/mL of ampicillin and grown to an OD_{600} of 0.5-0.7 at 37°C. Recombinant protein expression was induced using 0.5mM isopropyl-β-D-thiogalactopyranoside for approx. 3 hours. Cells were then lysed on iced using 0.32 mg/mL lysozyme with the presence of protease inhibitors. GST-fusion proteins were then attached to Glutathione Sepharose 4B beads that had been pre-washed in a binding buffer (50mM Tris pH 7.7, 1mM EDTA, 1mM DTT). Supernatant from lysed cells and Glutathione Sepharose were mixed by inversion at 4°C and then rinsed three times with binding buffer supplemented with 150mM NaCl. Suspensions of Sepharose beads with captured proteins were snap-frozen and stored at -80°C for future experiments.

2.4 Immunoblotting

Cell lysates were diluted with HEDM buffer (50mM HEPES pH 7.5, 1mM EDTA, 3 mM DTT, 10mM $MgSO_4$). GST-E-cadherin and GST-N-cadherin beads were added to lysates and interaction tubes were continuously inverted for 90 minutes at 4°C. Lysates were then centrifuged at 1300 x g for 3 minutes at 2°C and rinsed with HEDLM buffer (50mM HEPES pH 7.5, 1mM EDTA, 3 mM DTT, 10mM $MgSO_4$, 1% polyoxyethylene-10-lauryl ether). After the final rinse a solution of 4X buffer and 90mM DTT were added to lysates and incubated for 10 minutes in a 72°C water bath. Samples were then subjected to SDS-PAGE and immunoblotting using a primary antibody for the myc-tag and a secondary antibody (anti-mouse). Western blots were developed using AP buffer, BCIP, and NBT to compare binding affinities. The Coomassie Blue test was used as a control to verify that interactions were occurring between target proteins.

2.5 Preparation of Cell Lysates for Immunoprecipitation

HEK293 cells were grown in 10-cm dishes with serum-free DMEM and transfected with 10ug of plasmid DNA encoding an activated variant of Gα12 and Gα13 (containing a Q-to-L amino acid substitution that abolishes GTPase activity) using a solution of 2 mg/mL polyethylenimine (PEI). 40-44 hours after transfection, cells were rinsed with cold PBS, scraped, resuspended with lysis buffer (20mM Tris pH7.7, 150mM NaCl, 2mM EDTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM Na_2VO_4) that was supplemented with the protease inhibitors 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1.67 mM), leupeptin (2.1 mM), pepstatin (1.45 mM), Nα-tosyl-L-lysine chloromethyl ketone (58 mM), tosyl-L-phenylalanylchloromethane (61 mM), and phenylmethylsulfonyl fluoride (267 mM) and continuously inverted for 45 minutes. Lysates were then centrifuged at 16,000 x g for 30 seconds at 2°C. Supernatants were pipetted off and frozen for immunoprecipitation at -80°C.

2.6 Immunoprecipitation

A primary antibody for beta-catenin (Cell Signaling #9562) was added to cell lysates and tubes were continuously inverted for 24 hours. Protein A & G Sepharose was then added to each sample and tubes were rotated continuously for another 120 minutes. Lysates were then centrifuged at 16,000 x g for 30 seconds rinsed with cell lysis buffer three

times. After the final rinse and solution of 4X buffer and 90mM DTT were added to lysates and incubated for 5 minutes in a 95°C water bath. Samples were then split and subjected to SDS-PAGE and immunoblotting using a primary antibody for beta-catenin or a primary antibody for phosphor-tyrosine and a secondary antibody. Western blots were developed using AP buffer, BCIP, and NBT to reveal immunoprecipitation results.

3. Results

3.1 Protein Interaction Assays Reveal PTP1B binding with E-cadherin

Through the use of PCR-based mutagenesis, a plasmid containing the DNA sequence for PTP1B with the addition of a myc epitope was created. The myc epitope tag was used because the antibody for the myc sequence is more reliable in testing than the PTP1B antibody. Pulldown analysis with the use of GST/E-cadherin fusion beads was performed followed by immunoblotting with an anti-myc primary antibody and an anti-mouse secondary antibody revealed that binding does occur between PTP1B and E-cadherin. The band in the first column of Figure 4.A shows that PTP1B is being pulled down with the E-cadherin attached to the GST Sepharose beads, suggesting an interaction. The absence of a band in the GST column indicates that there is no interaction between PTP1B and the GST protein alone. The Coomassie Blue test was performed to ensure that all GST-fusion proteins in the experiment were equally apportioned in the samples.

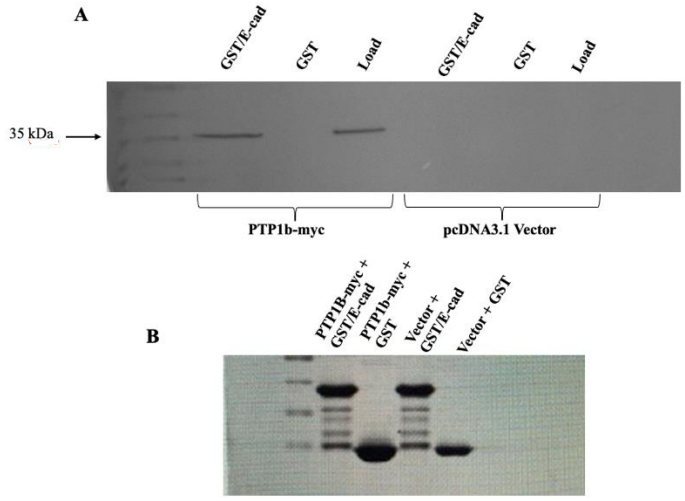
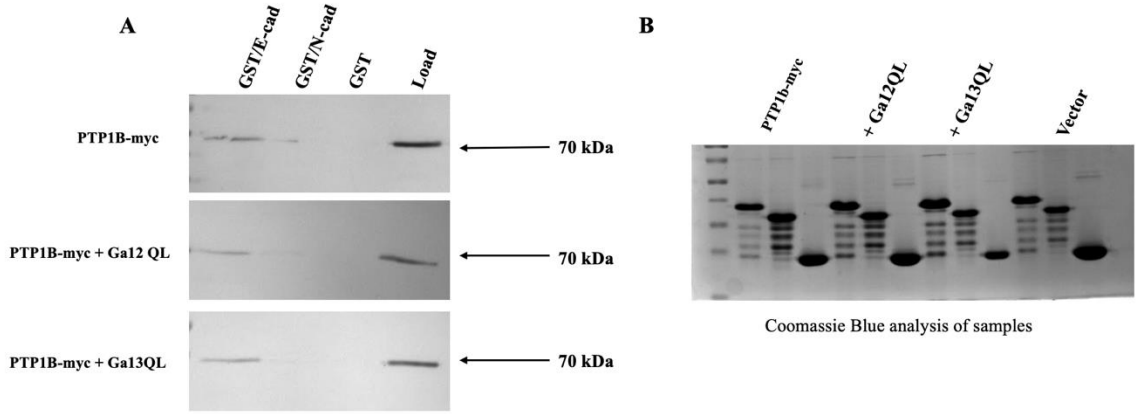


Figure 4. A) Immunoblotting shows binding between PTP1B and E-cadherin. pcDNA3.1 plasmid without the addition of PTP1B DNA sequence was used as a control vector. B) Protein levels for each sample were analyzed using Coomassie Blue staining.

3.2 Protein Interaction Assays with activated Gα12QL and Gα13QL Reveal Reduced Affinity for PTP1B

The next goal was to examine whether the interaction between PTP1B and cadherins is affected by Gα12/13 signaling. Cells were grown with PTP1B-myc plus the addition of Gα12QL or Gα13QL. Cell lysates were subjected to pulldown analysis with the addition of GST/E-cadherin and GST/N-cadherin beads and immunoblotting to detect co-precipitation of the myc-tagged PTP1B. Bands in Figure 5.A show decreased affinity for binding between PTP1B and E-cadherin when Gα12QL and Gα13QL are present, but not a total loss of binding. Bands in the GST/N-cadherin column show almost total loss of binding between PTP1B and N-cadherin when Gα12QL and Gα13QL are present. The Gaussian quantification data in Figure 5.C compares the ratio of pixels for each band compared to the load band. The ratios support the conclusion that there is reduced interaction between PTP1B and both cadherins. Figure 5 is a representative of multiple repetitions of the pulldown analysis and immunoblotting and results were consistent each

time. The Coomassie Blue test was performed to ensure that all GST-fusion proteins in the experiment were equally apportioned in the samples.



C

	pulldown/load ratio
E-cadherin + vector	0.402
E-cadherin + 12QL	0.239
E-cadherin + 13QL	0.249
N-cadherin + vector	0.085
N-cadherin + 12QL	pulldown not detected
N-cadherin + 13QL	pulldown not detected

Figure 5. A) Decreased affinity for E-cadherin binding when Gα12QL and Gα13QL are present. A blank vector lysate was used as a negative control. B) Protein levels for each sample were analyzed using Coomassie Blue staining. C) Gaussian Quantification data for the bands in part A.

3.3. Immunoprecipitation of Beta-catenin with Gα12QL and Gα13QL

A secondary goal was to examine whether beta-catenin would have increased phosphorylation due to the presence of signaling from Gα12 and Gα13. Cells were grown with the activated form of either Gα12QL or Gα13QL. Immunoprecipitation was performed on cell lysates with the addition of a beta-catenin antibody and Protein A&G Sepharose beads followed by Western blotting with either a beta-catenin primary antibody or a phospho-tyrosine primary antibody. Figure 6.A shows that beta-catenin was successfully immunoprecipitated with Ga12/13. Bands appear at the correct molecular weight for beta-catenin. Faint bands in the mock lanes are most likely due to the protein A&G Sepharose beads pulling down other proteins present in the lysates. A pre-rinse with the Sepharose beads could be done to try and pull out any random proteins before running the IP. Figure 6.B shows immunoprecipitation of beta-catenin with the use of the phospho-tyrosine antibody to detect elevated levels of phosphorylation on beta-catenin. There is no apparent increase in phosphorylation, because there are no bands showing up where we would expect the beta-catenin bands to appear. This is preliminary data, as these tests have only been repeated once.

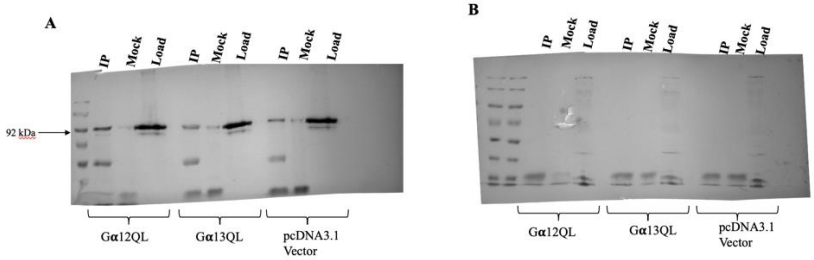


Figure 6. Immunoprecipitation of beta-catenin with G**α**12QL and G**α**13QL A) With beta-catenin primary antibody B) With phospho-tyrosine primary antibody

4. Conclusion

Cadherins have long been established as a key component for cellular adhesion. The G-proteins, Gα12 and Gα13, have been shown to interfere with the function of cadherins by binding to their cytoplasmic tail.[9,10] Which mechanisms have been disrupted because of Gα12/13 binding has not been established. The main goal of the current study was to determine if the predicted mechanism is disrupted when Gα12/13 bind to cadherins.

These methods have established that PTP1B binds to E-cadherin. Further tests have shown that in the presence of Gα12QL and Gα13QL there is a reduction of PTP1B binding to cadherins. This is promising data, that can be used to further test whether Gα12/13 is acting as a competitive inhibitor for PTP1B.

This is only the first step in determining if the predicted mechanism is one of the pathways towards beta-catenin dissociation and reduced cellular adhesion. More work will need to be done to test whether the presence of Gα12/13 will increase phosphorylation of beta-catenin and whether the tyrosine kinase, Src is the source for phosphorylation of beta-catenin.

5. Acknowledgements

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6. References

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