Defining the PIPKIγ707/SNX5 interaction and its possible association with E-cadherin

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E-cadherin, a cell adhesion molecule and tumor suppressor plays an important role in suppressing metastasis of cancers of epithelial origin by acting as the cornerstone of adherens junctions, which facilitate adhesion between epithelial cells[2]. The type I gamma phosphatidylinositol phosphate kinases (PIPKIγ) directly bind E-cadherin and produce phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$), a lipid messenger whose localized generation is necessary for E-cadherin transport and AJ formation[3]. The sorting nexins are a family of proteins that bind multiple phosphoinositides and function in endocytic and endosomal trafficking pathways via vesicles which internalize extracellular components[8]. We have identified a novel splice variant, PIPKIγ707 that associates with both E-cadherin and sorting nexin 5 (SNX5) independently *in vivo*. Data presented here demonstrate that amino acids 645-651 of PIPKIγ707's C-terminus are largely responsible for mediating the interaction between Iγ707 and sorting nexin 5. Results also indicate the possible existence of a PIPKIγ707/SNX5/ECD complex within epithelial cells, suggesting that PIPKIγ707 and SNX5 may play a role in the proper trafficking of E-cadherin.

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COVER SHEET

TITLE: **Defining the PIPKIγ707/SNX5 interaction and its possible association with E-cadherin**

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Defining the PIPKIγ707/SNX5 interaction and its possible association with E-cadherin

College of Agriculture and Life Sciences

Senior Honors Thesis

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Abstract

E-cadherin, a cell adhesion molecule and tumor suppressor plays an important role in suppressing metastasis of cancers of epithelial origin by acting as the cornerstone of adherens junctions, which facilitate adhesion between epithelial cells[2]. The type I gamma phosphatidylinositol phosphate kinases (PIPKIγ) directly bind E-cadherin and produce phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$), a lipid messenger whose localized generation is necessary for E-cadherin transport and AJ formation[3]. The sorting nexins are a family of proteins that bind multiple phosphoinositides and function in endocytic and endosomal trafficking pathways via vesicles which internalize extracellular components[8]. We have identified a novel splice variant, PIPKIγ707 that associates with both E-cadherin and sorting nexin 5 (SNX5) independently *in vivo*. Data presented here demonstrate that amino acids 645-651 of PIPKIγ707's C-terminus are largely responsible for mediating the interaction between Iγ707 and sorting nexin 5. Results also indicate the possible existence of a PIPKIγ707/SNX5/ECD complex within epithelial cells, suggesting that PIPKIγ707 and SNX5 may play a role in the proper trafficking of E-cadherin.

Introduction

Carcinomas or cancers of epithelial origin represent approximately 75% of all cancers[1]. E-cadherin, the epithelial isoform of the classical cadherins is a key cell adhesion molecule and tumor suppressor important for the establishment and maintenance of an epithelial morphology in order to suppress cancer metastasis. E-cadherin plays an important role in preventing metastasis by acting as the cornerstone of adherens junctions, which facilitate adhesion between epithelial cells[2,3]. Ling, et al. (2007) have shown that type Iγ phosphatidylinositol phosphate kinases directly bind to E-cadherin and drive E-cadherin trafficking to and from the plasma membrane through the generation of the lipid second messenger $PI4,5P_2$[3]. We have discovered a novel Iγ splice variant, PIPKIγ707, that is able to associate with E-cadherin as well as the cytoplasmic trafficking protein sorting nexin five (SNX5) *in vivo*. The role of the PIPKIγ707/SNX5 interaction in the cell is currently unknown. **We hypothesize that PIPKIγ707's unique C-terminus is mediating its interaction with SNX5 and that this interaction may play a role in the proper trafficking of E-cadherin.**

Background

E-cadherin is a major cell-adhesion molecule, a powerful tumor suppressor, and a key determinant of epithelial cell polarity[2]. Assembly of E-cadherin based adherens junctions (AJ) is obligatory for establishment of polarized epithelia and plays a key role in repressing the invasiveness of many carcinomas. AJs facilitate adhesion between epithelial cells at the basolateral plasma membrane[4].

The type Iγ phosphatidylinositol phosphate kinases (PIPKIγ) all directly bind E-cadherin, however only Iγ668 has been shown to facilitate E-cadherin trafficking[3]. PIPKIγ is a part of a family of enzymes responsible for the generation of phosphatidylinositol 4,5-bisphosphate ($PI4,5P_2$), a lipid messenger required by many signaling cascades, and whose localized generation is necessary for E-cadherin transport and subsequent assembly of AJ[3]. In addition, PIPKIγ undergoes alternative splicing of its C-terminus, resulting in the generation of four distinct splice variants each of which are suspected to play diverse roles in the cell (Figure 1). Each of the splice variants show distinct localization in the cell, which suggests specificity in

their cellular functions. Our laboratory hypothesizes that splice variant specific protein-protein interactions function as targeting factors for the localized generation of $PI4,5P_2$. The most well characterized splice variant, PIPKIγ668, binds to the cell adhesion molecule talin and facilitates its targeting to focal adhesions[7]. However, while PIPKIγ707 shares significant homology to the talin binding region of PIPKIγ668 it does not interact with talin (unpublished data, Schill 2008).

At present, the cellular function of PIPKIγ707 is unknown. To begin to define its function, a yeast-2 hybrid screen was performed using the unique C-terminal sequence of PIPKIγ707 as bait in order to identify interacting partners. The yeast-2-hybrid screen identified an interaction between PIPKIγ707 and cellular trafficking protein, sorting nexin 5 (SNX5). This interaction has been confirmed via co-immunoprecipitation and cellular co-localization as observed by immunofluorescence (Figure 2a+b). The direct interaction has also been confirmed by an *in vitro* pull down assay. The PIPKIγ707/SNX5 interaction has also been found to be specific for the PIPKγ707 splice variant only (Figure 2c).

Sorting nexins (SNX) are cytoplasmic proteins that function in endocytic and endosomal trafficking pathways via clathrin-coated vesicles which internalize extracellular components[8]. Once internalized, membrane proteins are sent to early endosomes where they are sorted and delivered to their cellular destinations. The SNX family of proteins is grouped based on the presence of a PX domain that binds different phosphoinositides, and two coiled-coil domains (CC), which facilitate protein-protein interactions and induce membrane curvature[8]. An interesting feature of SNX5 is its change in cellular localization in response to growth factor (GF) stimulation. In non-stimulated cells, SNX5 localizes to endosomal structures, but when stimulated with GF, SNX5 translocated to the plasma membrane[9]. This translocation to the plasma membrane upon GF stimulation may indicate a function in E-cadherin trafficking, particularly internalization. Interestingly, stimulation with epithelial growth factor (EGF) disrupts cell-cell adhesion and induces endocytosis of E-cadherin[5]. Preliminary results from our lab demonstrate that PIPKIγ707 directly interacts with SNX5 (*in vitro* and *in vivo)* and E-cadherin (Figure 2 and 3). The goal of the work presented here was to define the interacting region of PIPKIγ707 for SNX5. Also, since the generation of $PI4,5P_2$ by PIPKIγ is necessary for E-cadherin transport[3], we are interested in exploring a potential role for the PIPKIγ707/SNX5

complex in the trafficking of E-cadherin. Therefore, we hypothesize that PIPKIγ707's unique C-terminus is mediating its interaction with SNX5 and that this interaction may play a role in the proper trafficking of E-cadherin.

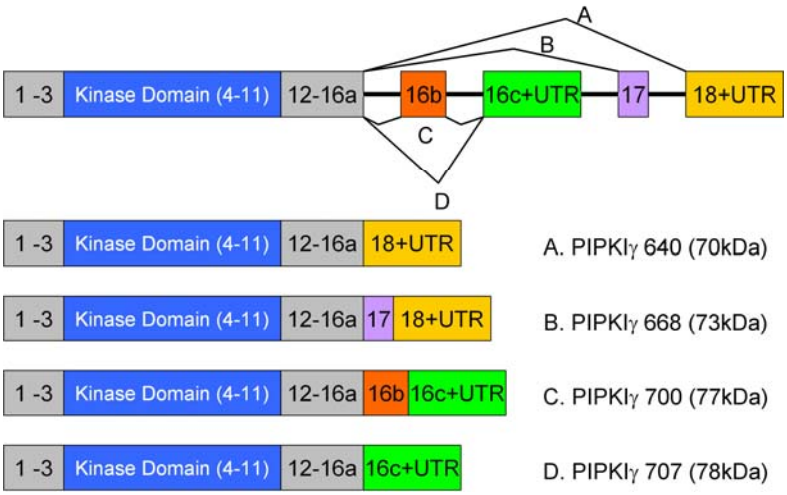


Figure 1. The C-terminus of the PIPKIγ's is alternatively spliced, generating 4 distinct splice variants. These four splice variants, PIPKIγ640, 668, 700, and 707 all have unique subcellular localization patterns and are thought to play diverse roles within the cell.

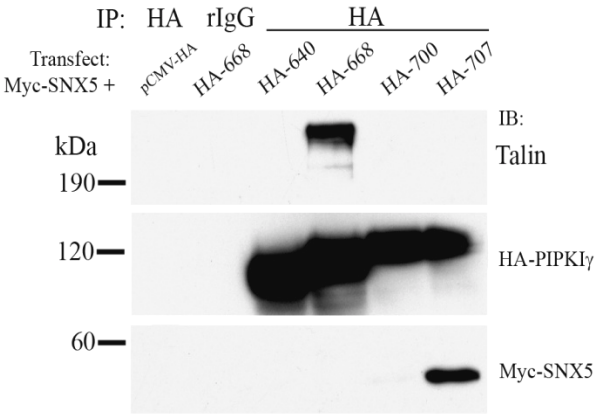
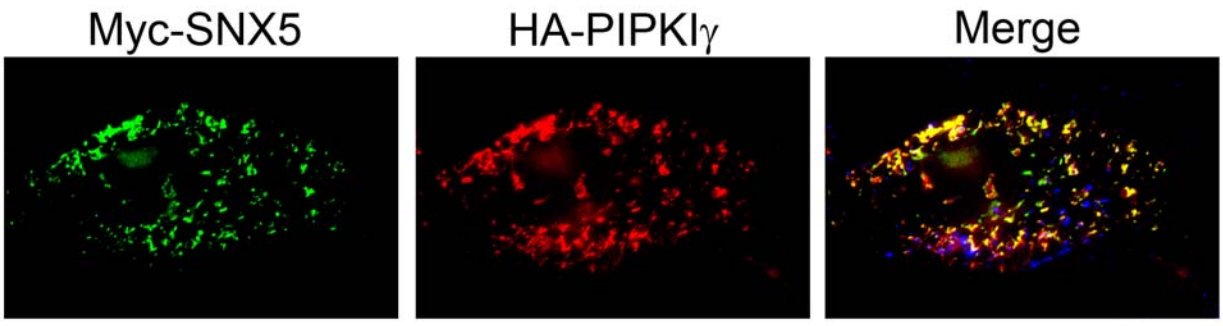
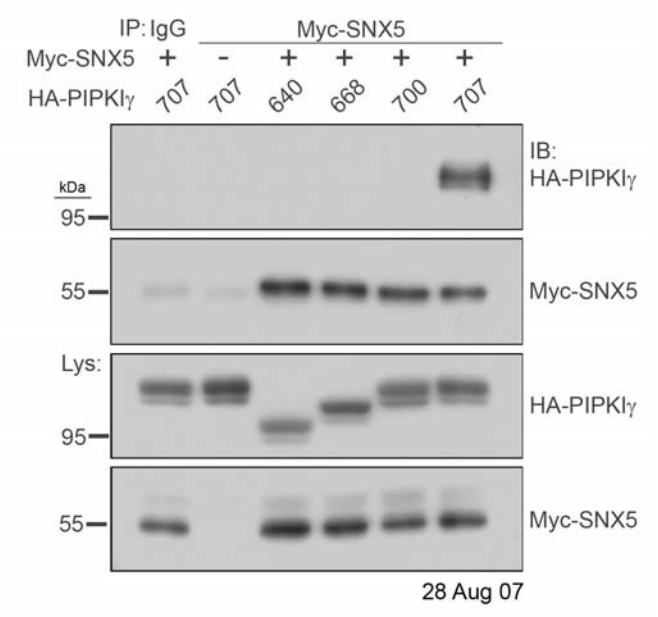


Figure 2. A) PIPKIγ707 specifically associates with SNX5. PIPKIγ707 and SNX5 were co-expressed in HEK-293 cells and HA-PIPKIγ was immunoprecipitated from the cell lysates. Myc-SNX5 was only detectable in the HA-PIPKIγ707 immunoprecipitate, indicating that the association between SNX5 and PIPKIγ is specific for Iγ707.
B) PIPKIγ707 and SNX5 partially co-localize in HeLa cells. HA-PIPKIγ707 and Myc-SNX5 were co-expressed in HeLa cells. Cells were fixed, stained, and visualized by indirect immunofluorescence. PIPKIγ707 and SNX5 were observed to strongly co-localize at structures peripheral to the plasma membrane, and to a lesser extent, punctuate cytoplasmic structures.
(Figures provided by Nick Schill)





C) PIPKIγ707 associates with SNX5 in vivo. The PIPKIγ splice variants and SNX5 were co-expressed in Hela cells. Myc-SNX5 was immunoprecipitated from the cell lysates. Only PIPKIγ707 was shown to associate with SNX5. (Figure provided by Nick Schill).

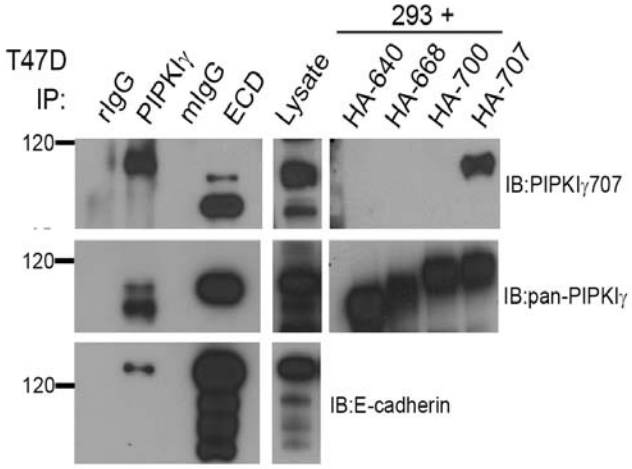


Figure 3: PIPKIγ707 associates with E-cadherin *in vivo*. Endogenous E-cadherin was immunoprecipitated from T47D cells, and the immunoprecipitates were probed with PIPKIγ splice variant-specific antibodies. Although the binding site of E-cadherin on PIPKIγ is thought to be the conserved kinase domain of all four splice variants, only PIPKIγ668 and PIPKIγ707 associate with E-cadherin in their endogenously. Interestingly, the molecular size of the major PIPKIγ707 polyclonal-reactive band is slightly smaller than its expected size. This suggests that although full-length PIPKIγ707 can associate with E-cadherin, the major associating PIPKIγ707 species has been modified in some fashion. (Figure provided by Nick Schill)

Materials and Methods

Constructs and Sequencing

Truncation mutants were generated by introducing a stop codon into the C-terminal coding region of the pCMV-HA/PIPKIγ707 construct via quickchange polymerase chain reaction (QC-PCR) (Stratagene). Truncations were made at amino acid residues 675 (Trp), 659 (Thr), 652 (His), and 645 (Tyr) in order to truncate the C-terminus beyond each stop codon site (Figure 4).

Once the binding region was sufficiently narrowed, conservative point mutations were introduced into this region using QC-PCR as described previously. Amino acid residues 650 (Pro) and 651 (Arg) were mutated to a leucine and amino acid residue 647 (Trp) was mutated to either a phenylalanine or a alanine. These conservative point mutations were chosen in order to conserve mass and size of the native residue, with the potential to disrupt binding. All mutants were fully sequenced with four internal PIPKIγ primers specific for pCMV-HA/PIPKIγ707 construct to confirm the truncation and point mutations.

Cell Transfection and Co-immunoprecipitation of Truncation and Point Mutants

HA-tagged serial truncation mutants and FL-pCMV-HA/PIPKIγ707 were transiently transfected with Myc-tagged SNX5 in Human Embryonic Kidney-293 cells (HEK-293) using Lipofectamine 2000 transfection reagent (Invitrogen). 24 hours post transfection, cells were lysed for 2 hours in SNX5 lysis buffer (100mM NaCl, 50mM Hepes, 5mM MgCl2, 0.5% NP-40, and Roche Mini Complete EDTA-free Protease Inhibitor Tablets)[10]. Wild-type or mutant PIPKIγ707 lysates were incubated with mouse anti-HA antibody (Covance) and protein G sepharose (Amersham) for 24 hours at 4°C while rotating, washed in SNX5 lysis buffer, and the protein was eluted in 5xSDS sample buffer. Immunoprecipitates were separated by SDS-PAGE, analyzed by Western blotting using mouse monoclonal antibodies specific for HA and Myc epitope tags, and detected using enhanced chemiluminescence (Pierce).

Cell Transfection and Immunoprecipitation of PIPKIγ707, SNX5, and E-cadherin

Madin-Darby canine kidney (MDCK)/pCMV-HA/PIPKIγ707 Tet-Off stable cell lines were incubated for 48 hours in the absence of doxycycline to induce expression of pCMV-HA/PIPKIγ707 protein. The PIPKIγ707 expressing cells were then transiently transfected with Myc-tagged SNX5 using Lipofectamine 2000 transfection reagent (Invitrogen). 24 hours post transfection cells were lysed in a slightly denaturing IP buffer (50mM Tris-HCl, pH 7.5, 1.0% Triton X-100, 0.5% DOC, 0.1% SDS, 50mM NaCl, 2mM EDTA, 2mM NaVanadate, and Roche mini-complete EDTA-free protease inhibitor tablets)[11] and sonicated at 11% amplitude 5x3 second bursts with one minute rests. Lysates were incubated with anti-ECD antibody (BD Biosciences) and protein G sepharose beads (Amersham) to immunoprecipitate endogenous ECD for 24 hours at 4°C while rotating. The immunoprecipitates were washed in lysis buffer and

eluted in 5xSDS sample buffer. Immunoprecipitates were separated by SDS-PAGE, analyzed by Western blotting using mouse monoclonal antibodies specific for HA and Myc epitope tags and endogenous ECD. The proteins were then detected by enhanced chemiluminescence (Pierce).

Results

PIPKIγ707 C-terminal amino acids 645-651 responsible for mediating SNX5 interaction

Upon introducing four truncation mutations into the C-terminus of PIPKIγ707, we found that truncation mutation PIPKIγ707Δ645 abolished SNX5 binding (Figure 3). PIPKIγ640, a PIPKIγ splice variant which does not contain the unique C-terminal tail present in PIPKIγ707, did not bind to SNX5 and served as a negative control for the truncation experiment (Figure 4). Therefore, amino acids 645-651 of PIPKIγ707's C-terminal tail are largely responsible for mediating the interaction with SNX5. In addition, truncation mutations PIPKIγΔ675, Δ659, and Δ652 appeared to have little to no effect on the binding of SNX5 (Figure 4).

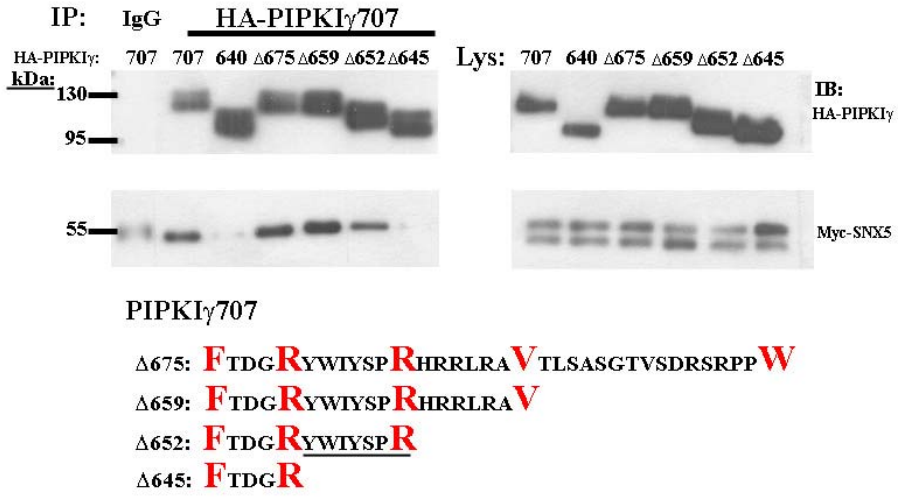


Figure 4: HEK-293 cells were co-transfected with WT HA-PIPKIγ707 or truncation mutants along with Myc-SNX5 and HA-PIPKIγ707 was IP'ed. Truncation mutants Δ675, Δ659, and Δ652 had no effect SNX5 binding. Truncation mutant Δ645 abolished SNX5 binding *in vivo*.

Point Mutations Trp647Phe, Trp647Ala, Pro650Leu, and Arg651Leu did not alter SNX5 binding to PIPKIγ707

PIPKIγ707/Trp647Phe, Trp647Ala, Pro650Leu, and Arg651Leu mutant PIPKIγ707 constructs were transiently transfected into HEK-293 cells. No visual differences in the binding of SNX5 were detected through the immunoprecipitation experiments using the mutant PIPKIγ707 C-terminus tails. Therefore, introduction of these four point mutations individually into the C-terminus of PIPKIγ707 did not significantly modify the binding ability of PIPKIγ707 for SNX5 (Figure 4).

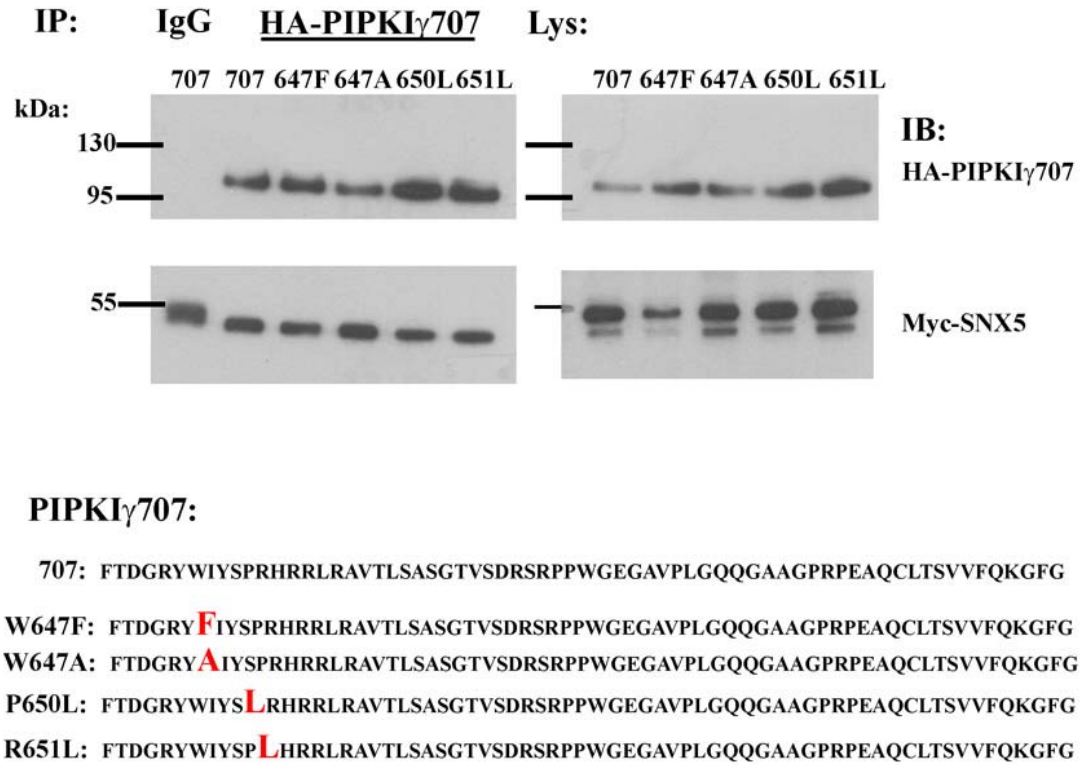


Figure 5: Point mutations Try647Phe, Try647Ala, Pro651Leu, and Arg651Leu in the C-terminus of PIPKIγ707 did not affect SNX5 binding. HEK-293 cells were co-expressed with mutant HA-PIPKIγ707 or wildtype along with Myc-SNX5. HA-PIPKIγ constructs were IP'ed and the point mutations did not affect SNX5 binding *in vivo*.

Possible Iγ707/SNX5/ECD Complex Formation

Preliminary results indicate that PIPKIγ707, SNX5 and E-cadherin form a complex within the cell. Upon transiently transfecting SNX5 into pCMV-HA/PIPKIγ707/MDCK-Tet On cells, an ECD IP detected Myc-SNX5 and HA-PIPKIγ707 proteins present in the immunoprecipitate

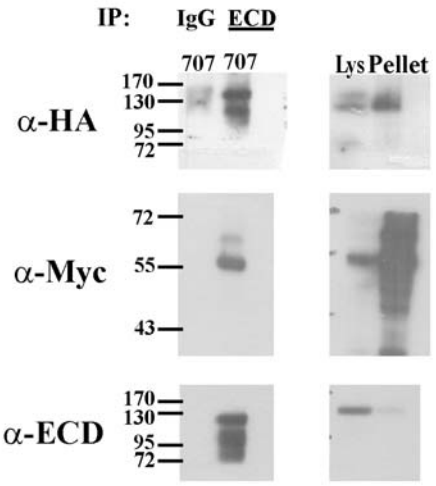


Figure 6: Preliminary results demonstrate a possible existence of PIPKIγ707/SNX5/ECD within the cell. MDCK-Tet-Off/PIPKIγ707-HA stable cells were expressed with SNX5-Myc protein and endogenous E-cadherin was IP'ed.

Discussion

The type I PIPKIγs undergo alternative splicing of their C-terminus generating four distinct splice variants which are homologous through PIPKIγ640 (Figure 1). PIPKIγ640, 668, 700, and 707 are thought to fulfill diverse cellular roles through interactions with unique binding partners. PIPKIγ707's C-terminus consists of a 67 amino acid extension that PIPKIγ640 does not possess. PIPKIγ707 is known to interact directly with SNX5, a cytoplasmic trafficking protein.

Interestingly, PIPKIγ640 does not bind SNX5 which indicates that PIPKIγ707's unique C-terminus may be responsible for mediating the SNX5 interaction. Data presented here demonstrate that introduction of truncation mutants PIPKIγ Δ675, Δ659, and Δ652 into PIPKIγ707's C-terminus did not disrupt SNX5 binding. Truncation mutant PIPKIγ Δ645 abolished SNX5 binding, indicating that amino acid region 645-651 of PIPKIγ707 plays a role in mediating the SNX5 interaction. Though amino acid region 645-651 has been identified as responsible for the SNX5 interaction, the nature of the interaction and the specific amino acids responsible have yet to be determined. Factors such as charge, size and structure must be considered when examining and mutating the interaction region. Interacting region 645-651 consists more specifically of amino acids Tyr-Try-Iso-Tyr-Ser-Pro-Arg which vary greatly in functional group structure/components.

Surprisingly, introduction of phenylalanine and alanine point mutations to residue 647 (Try) did not alter the binding ability of PIPKIγ707 for SNX5. These mutations served to eliminate the nitrogen containing ring structure (Phe) and the phenyl ring (Ala) of tryptophan. With the functional group of tryptophan containing a large phenyl ring conjugated to a nitrogen ring structure, we suspected that this amino acid may have played a role in mediating the interaction with SNX5. Residue 650 was mutated to a leucine which conserved the original mass, but disrupted the ring structure of proline. Reside 651 was converted to a leucine also which served to neutralize the basic charge present on arginine and conserve the original mass. Neither the Pro650Leu nor the Arg651Leu PIPKIγ707 mutants produced a change in binding affinity of SNX5. Though no visible change in binding occurred with the previous point mutations, further point mutations and other modification experiments of the 645-651 amino acid region may reveal the amino acids responsible for mediating the SNX5 interaction. An alternative explanation to these findings could be binding mediation based upon a two point protein contact region between PIPKIγ707 and SNX5, with one point of contact present on the C-terminal tail. Also, the interaction could be purely structural and simply mutating the tail may not provide much insight into defining the PIPKIγ707/SNX5 interaction region. Further analysis is necessary to fully conclude what amino acids are necessary and sufficient for binding SNX5.

Preliminary results also indicate possible complex formation between PIPKIγ707, SNX5, and E-cadherin with in the cell. A complex between PIPKIγ707, SNX5, and E-cadherin may reveal a mechanism for the proper trafficking of E-cadherin. As stated previously, E-cadherin is a powerful tumor suppressor that is known to interact with PIPKIγ668. PIPKIγ668 produces PI4, $5P_2$ which is necessary for E-cadherin transport and AJ formation[3]. Could the PIPKIγ707 interaction with E-cadherin and SNX5 be mediating E-cadherin trafficking as well? Could a PIPKIγ707/SNX5/E-cadherin complex complete another piece of the puzzle for cancer metastasis? While this data is exciting and intriguing, further analysis of this result is also needed to fully conclude the existence and physiological relevance of this complex.

Future Directions

Further analysis of the defined interacting region PIPKIγ645-651 is needed to specifically define the amino acids responsible for mediating the interaction with SNX5. Conservative point mutations still need to be made for the isoleucine and the serine of the interacting region. More conservative point mutations of the already examined amino acids may also reveal new binding information. Deletion mutations, multiple point mutations, and chimera analysis with the C-terminal tail may help to more specifically define the PIPKIγ707/SNX5 interaction. Protein structure analysis techniques such as x-ray crystallography and nuclear magnetic resonance could also be useful in discovering the folded structure of PIPKIγ707 and determining the exact manner and orientation of the SNX5 interaction.

Additional analysis of a possible PIPKIγ707/SNX5/ECD complex is needed to confirm its existence and role in the cell. To begin, the immunoprecipitation (IP) experiment needs to be repeated with controls for the expression of PIPKIγ707 and SNX5. To accomplish the proper controls, two additional IP's must be performed in accordance with the original E-cadherin IP. An IP must be performed where PIPKIγ707 expression is not turned on with in the cell, but SNX5 is over expressed and another must be performed where PIPKIγ707 expression is turned on, but SNX5 is not over expressed within the cell. If a PIPKIγ707/SNX5/ECD complex is confirmed through the procedure outlined previously, immunofluorescence experiments may be performed to confirm the co-localization of PIPKIγ707, SNX5, and E-cadherin within the cell.

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