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The Role of Phosphatidic Acid in PIPKI-regulated Actin Reorganization

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Akua Roach

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Abstract of the Dissertation

The Role of Phosphatidic Acid in PIPKI-regulated Actin Reorganization

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Production and breakdown of certain phosphoinositides plays key roles in a variety of cellular processes. Among these, phosphatidylinositol-4, 5-phosphate (PIP_2) is well known for its critical functions in membrane trafficking, cytoskeletal

organization, and signal transduction. Type I phosphatidylinositol-4-phosphate 5-kinase (PIPKI) is the main enzyme responsible for the synthesis of PIP_2. Although PIPKI activity is regulated by small G proteins and phosphatidic acid (PA), the contribution of these upstream regulators in actin cytoskeletal reorganization remains unclear. The binding region of some other PA-regulated proteins has been identified. From these, it appears that binding may either be direct or electrostatic in nature. I have identified basic residues in the proposed membrane-binding region of PIPKIγ that are required for membrane translocation, actin reorganization, and stimulation by PA. I also demonstrate here that the direct binding of PIPKI to PA through these residues is required for these important functions.

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List of Abbreviations

PIPKI	type I phosphatidylinositol-4-phosphate 5-kinase
PIP_2	phosphatidylinositol-4, 5-phosphate
PA	phosphatidic acid
PI(4)P (or PI)	phosphatidylinositol-4-phosphate
PC	phosphatidylcholine
kDa	kilodalton
PLD	phospholipase D
Arp2/3	actin related protein 2/3
N-WASP	neuronal Wiskott-Aldrich syndrome protein
DMEM	Dulbecco's modified eagle medium
PEI	polyethylenimine
mitoPLD	mitochondria-associated PLD
WT-PIPKI (or WT)	wild-type PIPKI
POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0-18:1 PC)
POPA	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate (16:0-18:1 PA)
TLC	thin layer chromatography
K_m	Michaelis constant
PM	plasma membrane
GFP	green fluorescent protein

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Acknowledgements

Firstly, I would like to thank my advisor, Guangwei Du, for taking a chance on me when I needed a new lab home and having patience as I transitioned from a biochemist to a molecular biologist. Thank you also to my supervisor, Mike Frohman, for his continued guidance, honesty, kindness, and concern in a field where most are not. In addition to Mike, thank you to the remainder of my Thesis Advisory Committee, Debbie Brown and Joav Prives, for their helpful comments and suggestions. Thank you also for the discussions which made me feel like a scientist and not just a student.

Thank you to the Pharmacology Department, some more than others, for creating a family atmosphere where I felt comfortable joking with established scientists, even though they were usually just making fun of me! A special thank you to Stella Tsirka for listening when I needed to vent or confide in someone and allowing me to be myself without passing judgment. Thank you to Bev Campbell for reassuring me that my feeling were justified given my unique situation and for going above and beyond to make sure I never went unnoticed.

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Last but definitely not least; I would like to thank my family. To my parents, Kwaku and Elizabeth, who instilled the importance of education and whose support got me through this difficult year. To my sister, Adwoa, the first “Dr.” in the family, for always trying to understand and offering sympathy when I struggled and encouragement when I waned. To my brothers, Kofi and Kwame, for all the laughs and friendly competition that allowed me to escape science and just be human and happy. To my husband, Glenn, who has been there from the very beginning with love, support, and understanding. His faith in me and constant encouragement has made the completion of this research possible. He has been my rock. Thank you to my son, Glenn Patrick, who has allowed me to put everything into perspective.

CHAPTER 1. INTRODUCTION

Type I phosphatidylinositol-4-phosphate 5-kinase (PIPKI) is the main enzyme responsible for the synthesis of the lipid second messenger PIP_2, which has critical functions in many cellular processes. The enzyme is regulated by phosphatidic acid (PA), small GTPases, and phosphorylation [1]. This work focuses on understanding the role and contribution of PA on PIPKI function, particularly in actin cytoskeletal reorganization.

PIPKI

PIP kinases are signaling regulated enzymes that phosphorylate membrane phosphatidylinositol phosphates to generate messenger phosphatidylinositol bisphosphates. There are three types of PIP kinase homologs: type I, type II, and type III. Type I PIP kinase (PIPKI) is both structurally and functionally different from both type II or type III PIP kinases (PIPKII and PIPKIII, respectively) [2, 3]. PIPKI phosphorylates PI(4)P to generate $PI(4,5)P_2$, or PIP_2. PIPKII phosphorylates PI(5)P and can be considered a PI(5)P 4-kinase and PIPKIII phosphorylates PI(3)P and can be considered a PI(3)P 5-kinase. Because PIPKI and PIPKII both generate PIP_2, they were first believed to have similar functions and compartment localizations [1, 4]. Data suggest that this is not entirely true. PIPKI regulates actin reorganization, secretion, and endocytosis [5-7]; PIPKII has been implicated in the regulation of granule secretion in platelets and aspects of tumor necrosis factor α-mediated signaling [8, 9]. PIPKI

is found primarily at the plasma membrane, nucleus, and Golgi. PIPKII is also found in the nucleus as well as the endoplasmic reticulum and actin cytoskeleton.

There are three PIPKI isoforms (α, β, and γ), each of which has different lengths, molecular weights, and tissue expression. PIPKIα has a molecular weight of 68kDa and PIPKIβ is 53kDa [10]. PIPKIγ has two previously described splice variants resulting in functionally unique C-terminal regions. The resultant proteins differ by a 26-amino acid extension, PIPKIγ635 and PIPKIγ661. The 661-variant has been shown to interact with the talin FERM domain in focal adhesions, regulates EGF (epidermal growth factor)-stimulated directional migration, basolateral targeting of E-cadherin, and endocytosis of the transferrin receptor [6, 11-16]. The 635-variant is the major contributor of the PIP_2 used in G-protein receptor-mediated IP_3 generation [17]. Two more human PIPKIγ splice variants, PIPKIγ700 and PIPKIγ707, have recently been described. Although their specific functions have not been assigned, they do posses distinctive cellular targeting and standard kinase activity [18]. The splice variant of PIPKIγ used in this study is PIPKIγ635, resulting in an 87KDa protein.

Although PIPKI α, β, and γ all seem to share the same biochemical activity and regulation *in vitro*, different functions have been implicated for each isoform in different systems. PIPKIα has been associated with tyrosine receptor kinase signaling, mRNA metabolism, and phagocytosis [19-23]. Enhancement of anaphylactic responses in PIPKIα knockout mice suggests a role for PIPKIα in the restraint of allergic reactions [24]. PIPKIβ has roles in platelet regulation. Knockout mice have decreased fertility and platelet disaggregation, suggesting a

role for PIPKIβ in platelet aggregation and adhesion [25]. PIPKIγ regulates synaptic vesicle trafficking and focal adhesions [6, 10-12, 26, 27]. PIPKIγ knockout mice die shortly after birth, perhaps due to defects in synaptic transmission and exo- and endocytosis [28].

All three PIPKI isoforms are also stimulated by PA and are regulated by phosphorylation and by the activity of small GTPases, such as Rho, Arf, and Rac [1]. Studies show that PIPKI can be activated either directly by Rho in actin remodeling or by its effector, Rho kinase, in neurite retraction [29]. Arf-dependent activation of PIPKI occurs at the Golgi and plasma membranes in regulation of vesicle trafficking [23, 30]. Rac has been shown to associate and regulate PIPKI. Together, Rac and PIPKI function by uncapping actin filaments, leading to increased actin assembly [31]. This effect requires both direct interaction and PIPKI activity. Phosphorylation of PIPKI has a general inhibitory effect on its activity. In yeast, phosphorylation of PIPKI by the casein kinase I homologue, CkiI, strongly inhibits its kinase activity [32]. In mammals, the initial kinase involved in phosphorylation has not yet been determined but one study suggests that protein kinase A may be involved [33]. PIPKI has been reported to undergo a depolarization-dependent dephosphorylation in neurons and has also been suggested to be tyrosine phosphorylated by focal adhesion kinase [6]. The regulation of PIPKI activity is both intricate and puzzling. It is not known which enzymes are involved or if there exists crosstalk between the pathways.

PA stimulates PIPKI activity but, interestingly, has no effect on PIPKII or PIPKIII [34]. The crystal structure of PIPKIIβ has recently been solved and a model

of its membrane-binding region has been generated [35] (Figure 1). The model of the widely flattened membrane-targeting region of PIPKIIβ suggests that there is a cluster of basic residues surrounding the interface of the dimerized enzyme. From the PIPKIIβ structure, the substrate binding pocket and proposed membrane associated region do not overlap; there are about 150 amino acids between them. An alanine/glutamate substitution within the activation loop in PIPKII and PIPKI has also been shown to be sufficient to swap their substrate specificities [3]. Although the PIPKII structure suggests a potential membrane interaction interface for PIPKI, it does not explain why PA has a clear stimulatory effect on PIPKI activity while having no effect on PIPKII.

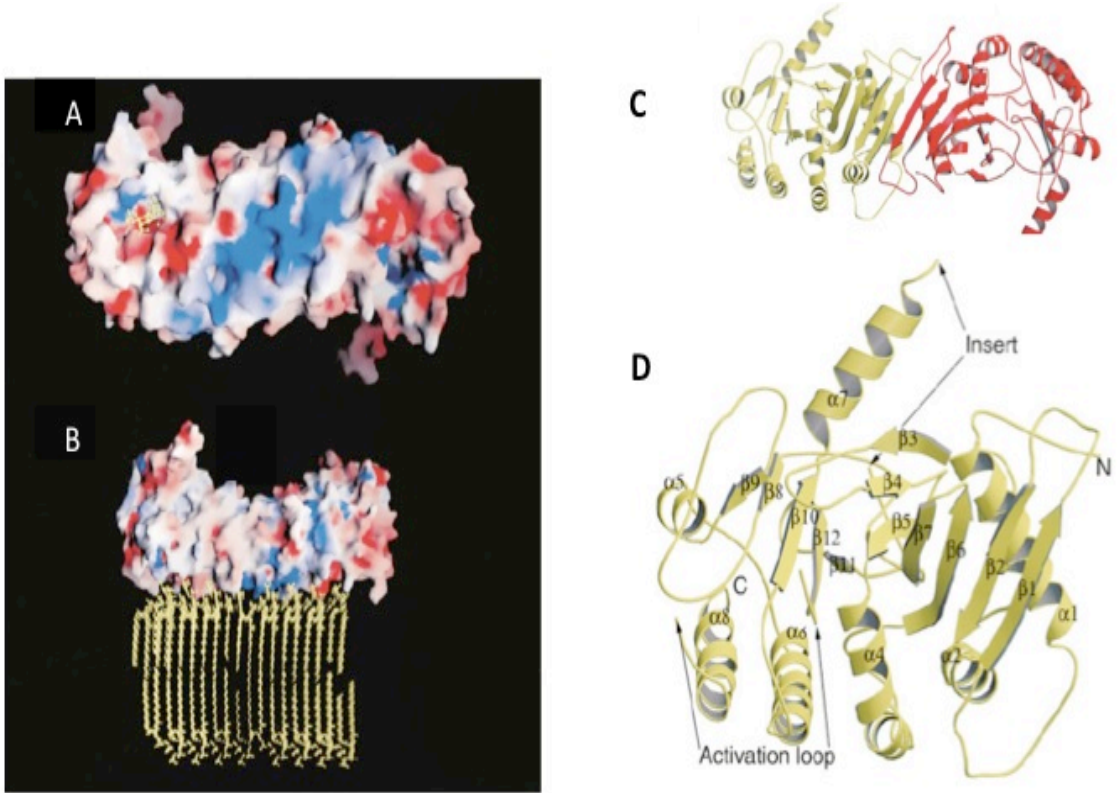


Figure 3: PIPKIIβ structure. A) Electrostatic structure. ATP is shown in yellow bonds docked into one of the two active sites. **B)** Docking of PIPKIIβ dimer onto a membrane surface. Charged residues are shown in blue (basic) and red (acidic). **C)** Ribbon diagram of PIPKIIβ dimer. One monomer is in yellow, the other in red**. D)** Ribbon diagram of one monomer. Beta sheets 1, 2, 6, and 7 of each monomer make up the widely flattened membrane-targeting region. The activation loop was disordered in the crystal structure and, therefore, could not be solved. From Rao, V, et al. Cell. 1998; 94(6):829-39

Phosphatidic acid and its interacting proteins

The membrane phospholipid phosphatidic acid (PA) is an important signaling molecule as well as a central intermediate for the synthesis and storage of lipids. Its metabolism and regulation play key roles in membrane biogenesis, cell proliferation, survival signaling, tumor progression, and differentiation [20, 36-38]. Some effects of PA are believed to act through membrane tethering, maintaining membrane structure and metabolism, and modulating enzymatic activity. Interestingly, although it is ubiquitously found in plant, animal, and fungal cells,

PA levels in resting cells are very low [38-40]. Stress, environmental cues, and other cellular signaling can transiently increase PA levels. The regulation of PA production and removal at specific subcellular localization are regulated by several enzymes. PA is produced mainly by phospholipase D (PLD) and diacylglycerol (DAG) kinase and can be removed by several enzymes with different modes of action [36, 37].

The mechanism through which PA regulates its target proteins is not yet clear. Many conserved modular protein domains, such as PX, PH, or FYVE domains, have been found to bind to different subcellular membranes through their interactions with specific phospholipids [41, 42]. Membrane targeting by these domains is critical for protein regulation, transportation, and signal transduction. However, not all proteins are targeted to membranes through these well-characterized lipid-binding domains. There are no recognizable conserved domains in these membrane association proteins. In these cases, electrostatic interactions between charged lipid head groups and proteins may be responsible for membrane targeting [43, 44]. Electrostatic interactions between membranes and proteins have been demonstrated for many proteins, including Src, K-Ras, and MARCKS, and are common regulatory mechanisms in cells (reviewed in [39]).

All identified PA-binding proteins are believed to interact with the negatively charged head group of PA lipids through positively charged amino acid residues on these proteins. This interaction is mediated by the known conserved domains in some proteins, such as the PH domain of Sos and the PX domain of $p47^{phox}$ [45, 46], or by a motif rich in basic amino acids in other proteins

(*i.e.*, Raf-1, DOCK2 and SHP-1; Table 1) [47-49]. The negatively charged phosphate head of PA is perhaps recognized by a positively charged site (consisting of Lys, Arg, and/or His residues) on PA-binding proteins [39, 44, 48, 50]. These sites were identified by mutating basic residue candidates, usually identified based on a crystal structure or other structural clues, to either alanine or anionic residues. PA has also been shown to directly regulate the activity of these proteins [34, 48, 50]. Analysis of deletion and site-specific mutants of these proteins suggests that perhaps a structural fold, localized charge, or electrostatic interaction rather than a universal conserved sequence is responsible for PA recognition [44, 50-53].

Table 3: Phosphatidic acid binding proteins and their identified PA-binding regions. Underlined are the specific residues that have been shown to have more of an effect on PA-binding than the nearby residues. All basic residues are highlighted in orange.

Protein	Sequence	Citation
Sos-PH	HERHIFLFDGLMICCKSNHGQPRLPGASN	Nat Cell Biol **9**,706-12 (2007)
p47^{phox}-PX	VYRRFTEIYEFHKTLKEMFPIEAGAINPENRIIPHLPAPKWFD	Embo J **21**, 5057-68 (2002)
Raf-1	FRNEVAVLRKTRHVNILLFMGYMTKDNLAIVTQWCEG	J Biol Chem **275**, 23911-8 (2000)
DOCK2	EYGVREMPDFEDRRVGRPRSM (60) RSKKRT	Science **324**, 384-7 (2009)
SHP-1	SSKHKEDVYENLHTKNKREEKVKKQRSADKEKSKGSLKRK	Biochemistry **38**, 11993-2002 (1999)
mTOR	YGRDLMEAQEWCRKYMKSGNVKDLTQAWDLYYHVFRRISKQ	Science **294**, 1942-5 (2001)

Although simple electrostatic interactions appear to be a general mechanism for PA-protein interactions, some proteins are specifically regulated by PA, but not by other acidic phospholipids like phosphatidylserine (PS) and PIP_2. It is still not clear how this specificity is achieved but it might be a way to ensure signaling specificity in cells. Here, I identify a basic cluster of specific residues in the proposed membrane-binding region of PIPKIγ that is required for actin reorganization.

PIP_2 Regulation of Actin Reorganization

Rapid actin cytoskeleton reorganization is an important cellular mechanism associated with membrane dynamics that can provide the force for cellular processes such as cell migration, cell fusion, and exo- and endocytosis [54, 55]. The structure and regulation of the actin cytoskeleton are controlled by a large number of actin-binding or actin-related proteins. Many proteins like the Arp2/3 complex, are involved in nucleation of new actin filaments whereas others enhance filament severing and depolymerization [54]. Polymerization is further regulated by proteins that interact with actin monomers or filament barbed end, including proteins involved in actin filament bundling and cross-linking to achieve three-dimensional actin structures.

Because actin reorganization is important for so many crucial cellular processes, it is controlled by various signaling pathways. Small GTPases and phosphoinositides have been well studied in their roles to mediate proper spatial and temporal regulation of cytoskeletal actin [55]. PIP_2 interacts directly with many

actin-binding proteins. It promotes the formation of actin filaments at the plasma membrane by activating proteins involved in filament assembly while inhibiting proteins involved in disassembly.

N-WASP (neuronal Wiskott-Aldrich syndrome protein) simultaneously binds several PIP_2 molecules which activate the protein at the plasma membrane [54-56]. Arp2/3 then binds N-WASP and is itself activated. Because Arp2/3 creates branched actin networks at the PM by nucleating new filaments while anchored to preexisting ones, it is believed that an increase in local PIP_2 density can activate the N-WASP/Arp2/3 actin polymerization pathway at the membrane [54, 57].

As aforementioned, PLD2 hydrolyzes the abundant membrane lipid PC to PA. PA, in turn, is a key regulator of PIPKIγ activity. In the presence of PA, PIPKIγ production of PIP_2 is greatly increased, which may result in an increase of local PIP_2 density at the membrane. Coupled with the finding that even a small change in PIP_2 concentration can activate N-WASP-mediated actin polymerization, it is not surprising that regulation of PIPKIγ by PA at the plasma membrane is an important regulator of actin reorganization.

This dissertation describes how I identified basic residues in the proposed membrane-binding region of PIPKIγ that are required for membrane translocation, stimulation by PA, actin reorganization, and direct binding to PA.

CHAPTER 2: A BASIC CLUSTER OF RESIDUES IN PIPKIγ IS NECESSARY FOR ITS PLASMA MEMBRANE LOCALIZATION

PA binding by many PA-binding proteins is known to be mediated by polybasic amino acid regions (reviewed in [44]). PIPKIγ does not have a typical lipid-binding module, such as a PH domain, suggesting its activity is regulated by PA through the binding of basic amino acids [41, 44]. Because all 3 isoforms of PIPKI are activated by PA, a conserved pattern of basic residues might be present amongst the PIPKI isoforms but absent from PIPKII, which is not stimulated by PA, that may stabilize PIPKI at PA-rich membranes.

The PIPKI substrate, PI(4)P, is a membrane lipid found primarily in the inner layer of the plasma membrane and Golgi. PIPKIγ is known to be recruited to the plasma membrane but the residues involved in this recruitment have yet to be identified. Because its substrate can be found at the plasma membrane, plasma membrane localization is important for PIPKIγ activity, which leads to PIP_2 production, and ultimately actin reorganization.

Others have shown that PIPKIγ can be chaperoned to the membrane by directly binding proteins which themselves are membrane localized [6, 11, 12]. For example, one of the PIPKIγ splicing isoforms, PIPKIγ-90 binds the FERM domain of talin, a cytosolic protein that links integrins to the actin cytoskeleton, and is recruited to sites of focal adhesions at the plasma membrane [6, 11]. I have identified four amino acids within the proposed membrane binding region of PIPKIγ that are important for membrane localization. Although I cannot rule out

the possibility here that these residues may be important for the binding of PIPKIγ to yet some unknown chaperone, I later show that these residues directly interact with membrane lipids.

Materials and Methods

Prediction Software Primary sequence alignment of PIPKIIβ and the α, β, and γ isoforms of PIPKI were performed using MultAlin [58]. Secondary structure predictions were performed using the programs nnPredict (Expasy Tool) and Phyre [59].

Construction of GFP constructs Wild-type GFP tagged PIPKIγ was received as a gift from Dr. Gilbert Di Paolo at Columbia University. Site-directed mutagenesis using *Pfu Turbo* (Stratagene) was used to generate the mutants used in this study according to manufacturer's protocol. Because the target residues are spaced apart, a two-step mutagenesis was employed where WT-PIPKIγ is used as a template to mutate 1 or 2 basic residues before the resultant construct is used to mutate the remaining residues to alanines. Primers and templates used in this study are listed in Table 2. Generated constructs were verified by DNA sequencing using sequencing primers 5'-GATTACTTGTACTCCCTGTGC-3' and 5'-CCGTGTTGCTCATGAAC-3'.

Table 4: Construct primers and templates. Those used to generate the GFP-tagged constructs used in this study are listed below. Only the forward primers are listed below, all reverse primers are the reverse complement of the forward sequences.

Construct	Template	Primers
A (KRHH)	Step 1: pEGFP-hPIPKIγ (WT) Step 2: Product from Step 1	Step 1: 5'-GTG GGC CAC CTG AGC TCC GCG CCC GAA GCC GAC GTG CTC ATG CAG-3' Step 2: 5'-C AGC AAC CTC ACC CCC GCC CGC CGC TTC CAG GAC TTC CGC TTC-3'
B (HKK)	pEGFP-hPIPKIγ (WT)	5'-G ACC GTC ATG GCC GCG GAG GCC GAG TTC CTG CAG GCG CTG CTC CC-3'
C (RKKR)	Step 1: pEGFP-hPIPKIγ (WT) Step 2: Product from Step 1	Step 1: 5'-C CTC AAC CAG AAC CCG GCG ACG CTG CTG CCC GCG TTC TAT GGG CTG TAC TG-3' Step 2: 5'-GTG CAG TCG GGG GGC GCG AAC ATC GCC GTC GTG GTC ATG AAC-3'
A + B	pEGFP-**A**	5'-G ACC GTC ATG GCC GCG GAG GCC GAG TTC CTG CAG GCG CTG CTC CC-3'
B + C	pEGFP-**C**	5'-G ACC GTC ATG GCC GCG GAG GCC GAG TTC CTG CAG GCG CTG CTC CC-3'
C + A	Step 1: pEGFP-**C** Step 2: Product from Step 1	Step 1: 5'-GTG GGC CAC CTG AGC TCC GCG CCC GAA GCC GAC GTG CTC ATG CAG-3' Step 2: 5'-C AGC AAC CTC ACC CCC GCC CGC CGC TTC CAG GAC TTC CGC TTC-3'
KR	pEGFP-hPIPKIγ (WT)	5'-GTG GGC CAC CTG AGC TCC GCG CCC GAA GCC GAC GTG CTC ATG CAG-3'
HH	pEGFP-hPIPKIγ (WT)	5'-C AGC AAC CTC ACC CCC GCC CGC CGC TTC CAG GAC TTC CGC TTC-3'
PM-**A**	pEGFP-**A** & pPM-mcherry	CMV-F-NdeI: 5'-CTTGGCAGTACATCAAGTGTATC-3' PM-R-AgeI: 5'-TGATACCGGTGCGTCTTTACGCTTAGACTTTA TAC-3'

Cos7 cell culture, transfection, and immunofluorescent staining Cos7 cells were grown in Dulbecco's modified eagle medium (DMEM) containing 10% bovine calf serum and antibiotics. Cells were maintained at 37^{o}C in 5%CO_2. Early on in my transfection experiments, I noticed that the WT transfected cells were uncharacteristically rounded and often floated off the culture plates and coverslips. Perhaps the lipid-based transfection reagents that were being used

lead to rapid plasmid DNA uptake, causing expression of too much protein in a relatively short time. In addition, lipid-based transfection may also cause the change of membranes. PIPKI is involved in cell shape dynamics and actin reorganization [21, 23, 60, 61]. Too much PIP2 production may inhibit the dynamic changes of actin and membranes. This would cause the otherwise adhesive cells to round up and detach from the plates or coverslip, especially when washed. The expression from transfected DNA is slower and more uniform in different cells using the linear polyethylenimine (PEI) transfection reagent while similar transfection efficiency is achieved compared to the lipid-based reagents. A time-course experiment revealed that PEI produced sufficient PIPKI expression (see Figure 2.1) with minimal cell shape deformities (qualitative analysis) at an 18-hour time point. Thus all transfections described in this dissertation use PEI for 18-hour transfections. Semiconfluent (30%) cultures were grown on glass coverslips one day prior to transfection with GFP-tagged PIPKIγ constructs using Polyethylenimine (PEI) 22K (gift from Dr. Jen-Chih Hsieh). 18hr after transfection, cells were fixed in 2% paraformaldehyde, permeabilized, and incubated with rhodamine-conjugated phalloidin (Invitrogen, 1:200 dilution). Confocal images were captured using a Leica SP2 Confocal Microscope.

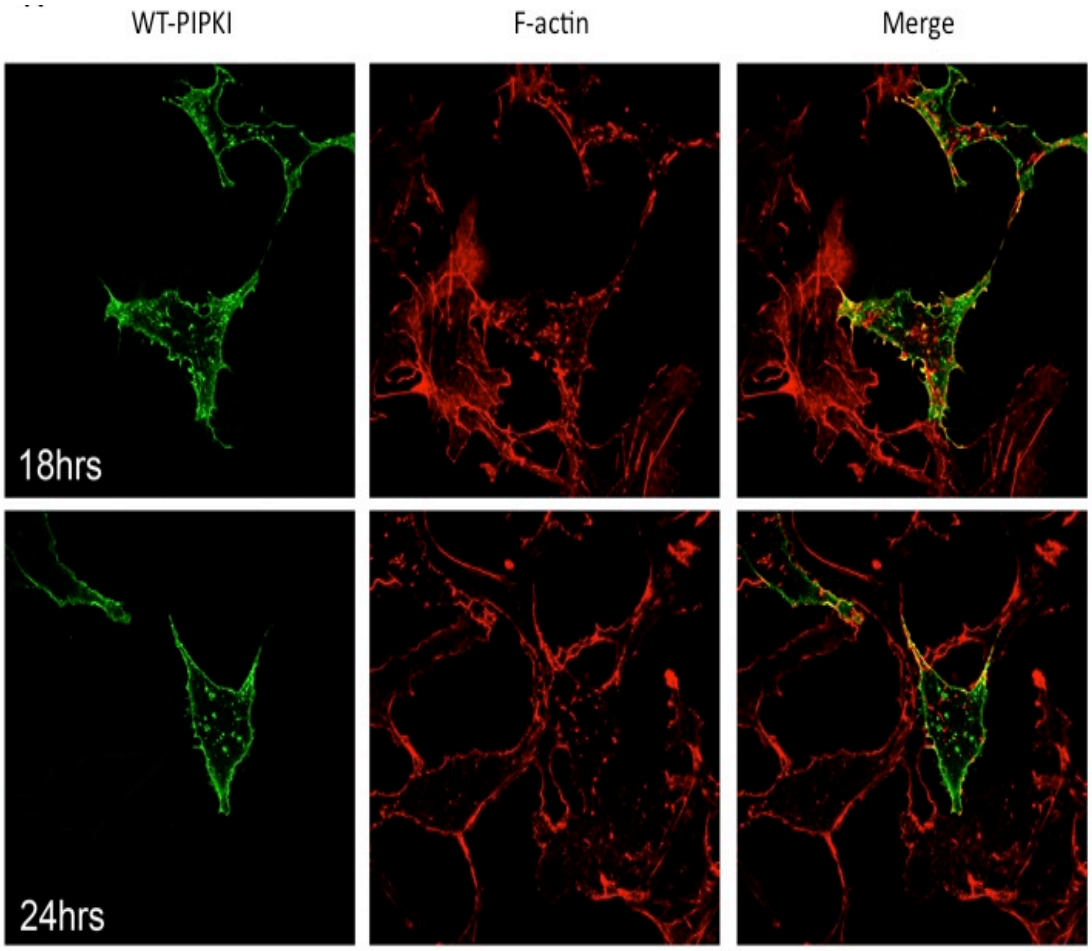


Figure 4.1: Optimization of transfection condition. Cos7 cells were transfected with WT-PIPKIγ and PEI transfection reagent for 12, 18, and 24 hours before fixing and staining for imaging. Data not shown for 12-hour time point, PIPKI expression cannot be detected. Most transfected cells rounded up and died 36 hr after transfection. Liposome-based transfection reagents are less consistent due to high toxicity. F-actin was stained using rhodamine-phalloidin.

3T3 mitoPLD-inducible cell culture, transfection, and staining mitoPLD-inducible 3T3 cells were grown in Dulbecco's modified eagle medium (DMEM) containing 10% bovine calf serum and antibiotics. Cells were maintained at 37^{o}C in 5%CO_2. Semiconfluent (30%) cultures were grown on glass coverslips and induced with mifepristone 6 hrs prior to transfection. Cells were transiently transfected with GFP-tagged PIPKIγ constructs using Polyethylenimine (PEI) 22K. 18hrs after

transfection, cells were fixed in 2% paraformaldehyde, incubated with MitoTracker Deep Red FM (Invitrogen), then permeabilized before staining with rhodamine-conjugated phalloidin (Invitrogen). Confocal images were captured using the Leica SP2 Confocal Microscope.

Results

The primary sequences of PIPKIIβ and all three isoforms of PIPKI (α, β, γ) were aligned using Multilink in order to identify the basic amino acids present in PIPKIs, which binds PA, but absent in the membrane association region of PIPKIIβ, which does not bind PA, (Figure 2.2A,B). By also comparing secondary structure predictions of PIPKIγ, performed by Expasy and Phyre softwares, with the solved secondary structure of PIPKIIβ, I discovered that the secondary elements in the proposed membrane-binding region (alpha helix 4 and beta strands 1, 2, 6, and 7) were likely conserved between the two enzymes. I identified candidate basic amino acids at or near the proposed structure elements in PIPKIγ that met the following requirements: 1) the basic residues in PIPKIγ must align with uncharged or negatively charged residues in PIPKII, 2) the residues had to be within the proposed membrane binding region, and 3) they must be at or close to the nearest conserved secondary element.

A

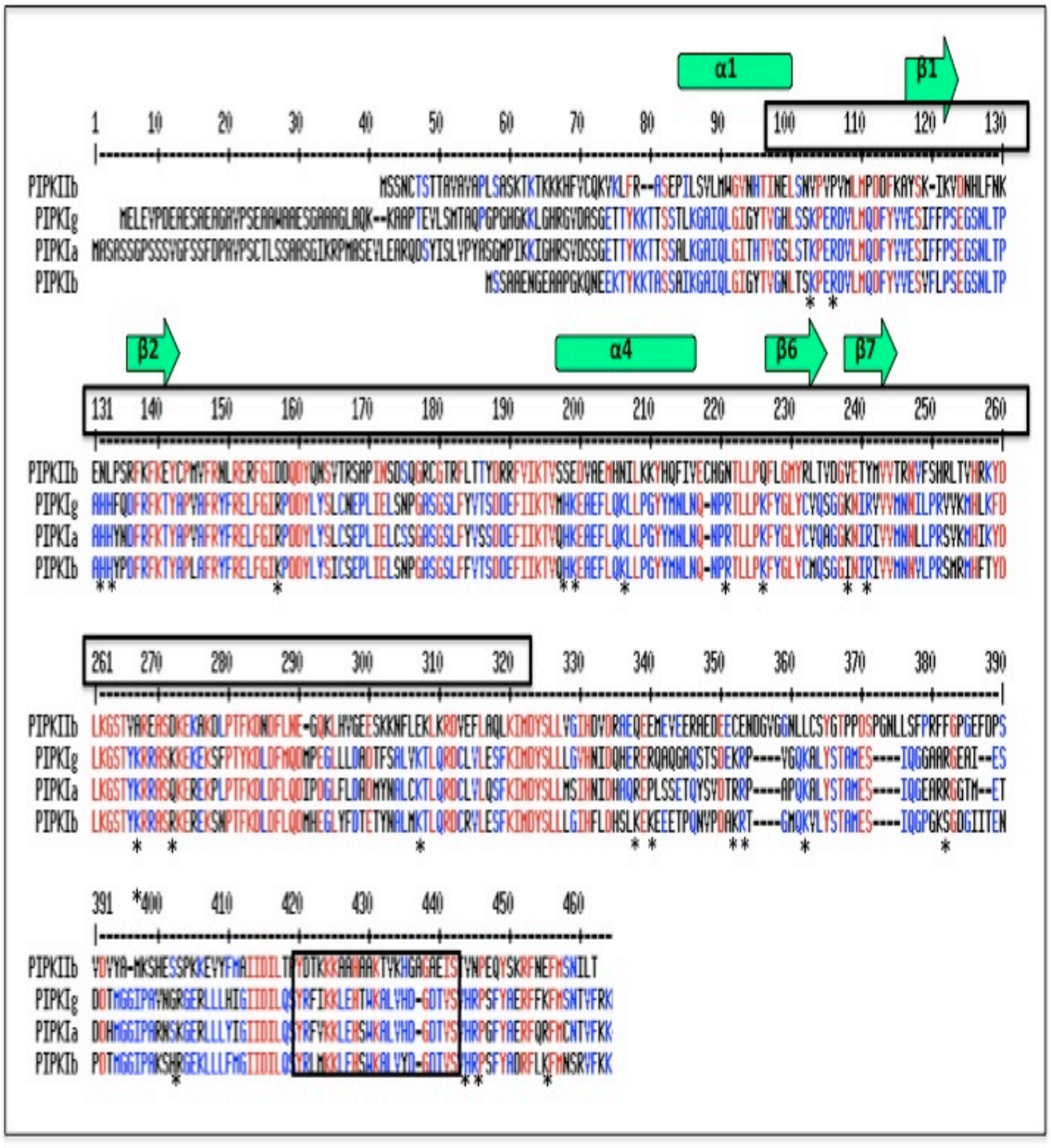


Figure 2.2A Sequence alignment of PIPKI isoforms (α, β, γ) and PIPKIIβ. Conserved amino acids are highlighted in blue (75% conserved) and red (100% conserved). Boxed numbers indicate conserved kinase core domain. Amino acids in the activation loop are boxed. Green arrows and bars indicate secondary structure elements (beta strands and alpha helices, respectively) and corresponding amino acids for PIPKI predicted using Expasy and Phyre prediction software which also correspond to the same PIPKII secondary structure elements from the solved structure implicated in membrane targeting.

Based on both primary and secondary structure analysis, candidate PIPKIγ basic residues, not found in PIPKIIβ, were identified within the kinase domain that were grouped and mutated to alanines to generate our 'charge mutants,' **A**, **B**, and **C** (Figure 2.2B,C). To determine whether overall protein charge was responsible for PIPKIγ regulation, these mutants were also grouped to generate 'grouped mutants' **A+B**, **B+C**, and **C+A** where seven or eight basic residues were mutated to alanines, resulting in mutants with decreased charge at the proposed membrane-binding region (Figure 2.2B,D).

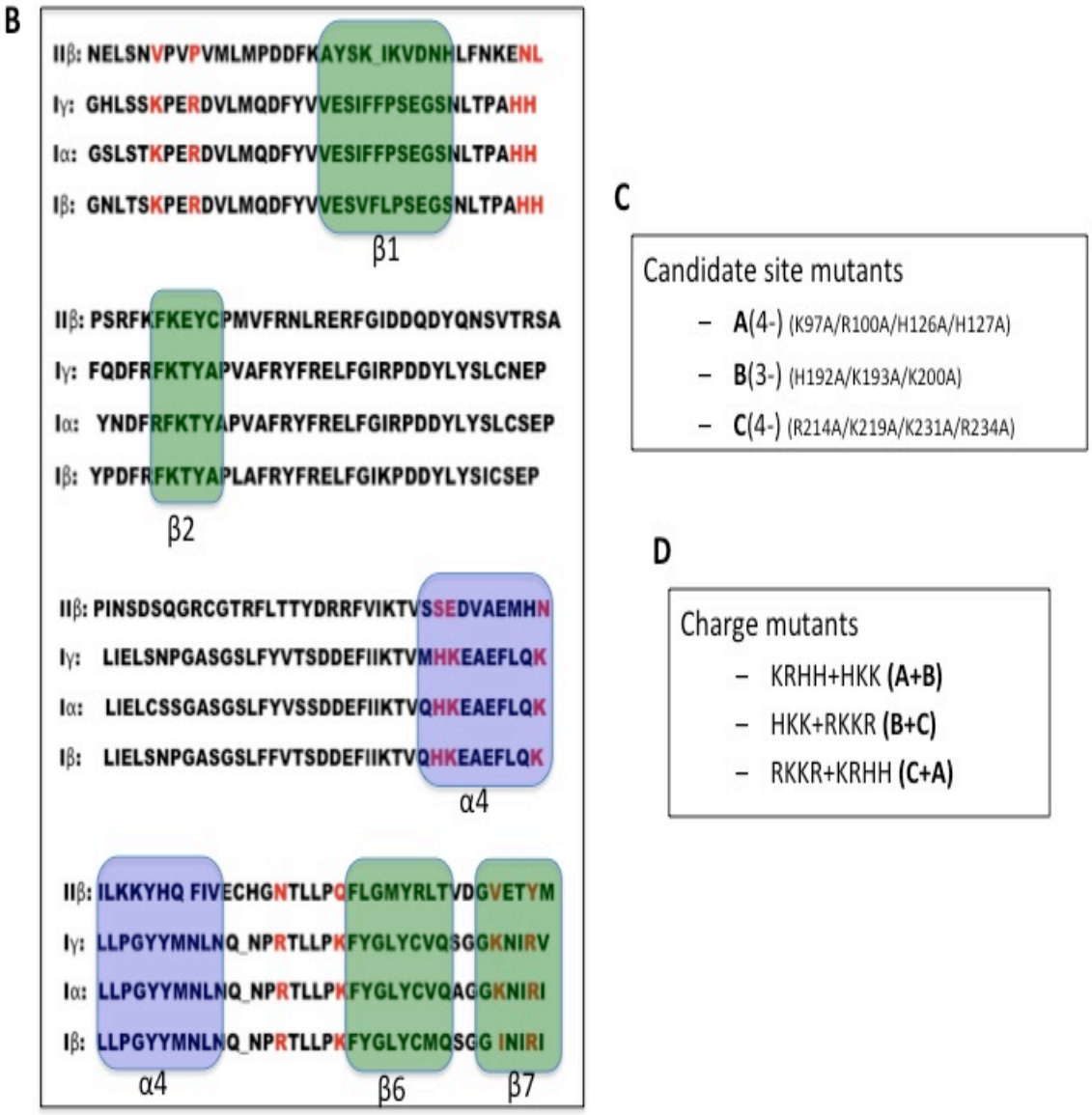
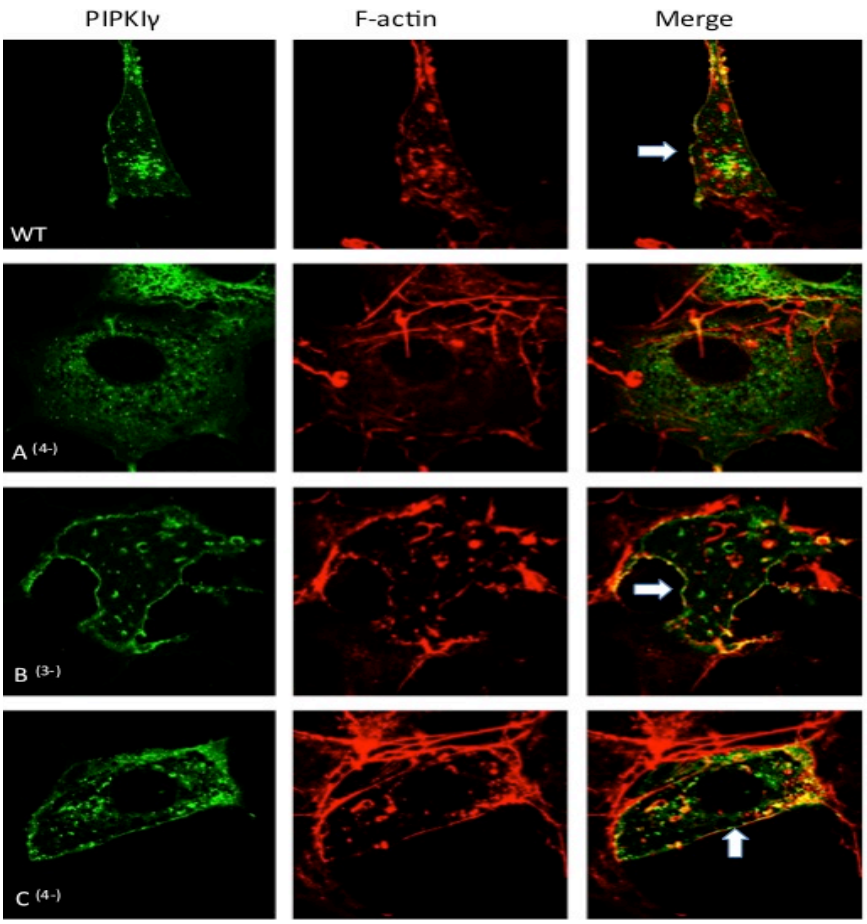


Figure 2.2(B-D): Generation of PIPKIγ mutants. B) Sequence alignment of the proposed membrane-binding region of PIPKIIβ and the corresponding PIPKI isoform (α, β, γ) residues. Green and purple bars indicate secondary structure elements (beta strands and alpha helices, respectively) conserved between the Type I and Type II enzymes, as predicted by prediction software. Highlighted in red are the 11 basic residues conserved in the three PIPKI isoforms that are absent from the Type II enzyme at or near our secondary elements of interest. **C)** Candidate site mutants were generated by mutating each residue in PIPKIγ from the corresponding basic residue to an alanine. Groupings are shown in the above panel. **D)** Charge mutants were generated by combining the candidate site mutants in all possible combinations to generate mutants that had a decrease in the overall charge (by -7 or -8) of the proposed membrane-binding region.

To determine whether basic residues in the proposed membrane-binding region are involved in PIPKIγ plasma membrane localization, Cos7 cells were transfected with GFP fusion constructs of the PIPKIγ mutants (Figures 2.3A, C). As expected, WT-PIPKIγ localized to the plasma membrane and dispersed vesicles. The **B** and **C** mutants also localized to the plasma membrane; the **C** mutant showed slightly reduced plasma membrane localization, having still a noticeable amount of protein remaining in the cytoplasm and some cells with no protein at the membrane at all. Interestingly, the **A** mutant was unable to remain on the plasma membrane and was cytoplasmic. Graphical data of images are of a qualitative assessment.



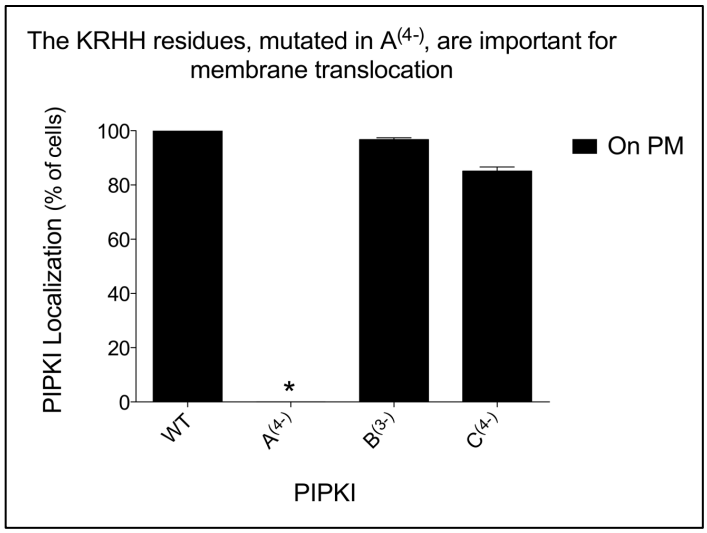
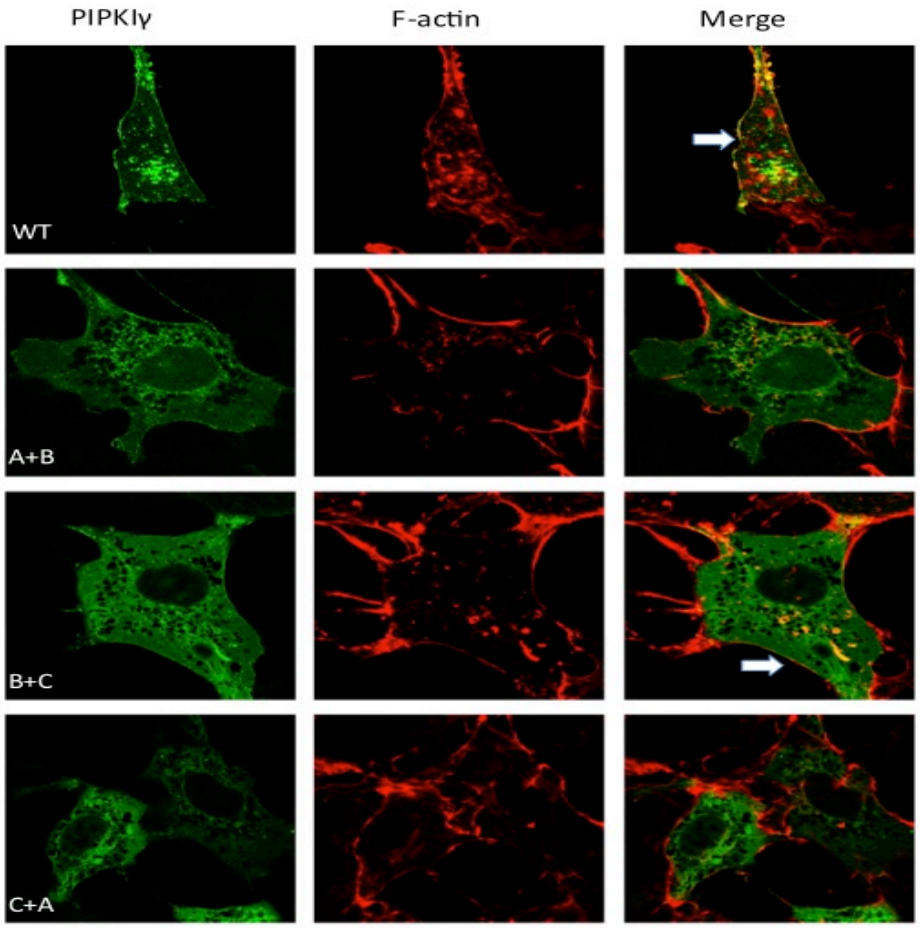


Figure 2.3A. The A mutant exhibits decreased membrane localization. Confocal images of Cos7 cells transfected with GFP-PIPKIγ charge mutant constructs. Plasma membrane (PM) Localization of the B and C mutant constructs show a pattern similar to that of the WT protein, suggesting that these mutants do not greatly affect membrane localization although the C mutant shows reduced localization. The A mutant causes a dramatic decrease in membrane localization. Qualitative quantification on bottom panel (50 cells counted each, N=3). F-actin was stained using rhodamine-phalloidin. Arrows point to membrane localized protein. T-test *p<0.01.

To investigate whether the overall charge of the large basic area of the proposed membrane-binding region is responsible for membrane recruitment, the grouped mutants were transfected into Cos7 cells, graphical data of images are of a qualitative assessment. (Figures 2.3B,C). Interestingly, the two mutants containing the **A** group of mutated amino acid residues were unable to localize to the plasma membrane. The **B+C** mutant was mainly on the plasma membrane. This result suggests that charge alone is not sufficient to recruit PIPKIγ to PM.



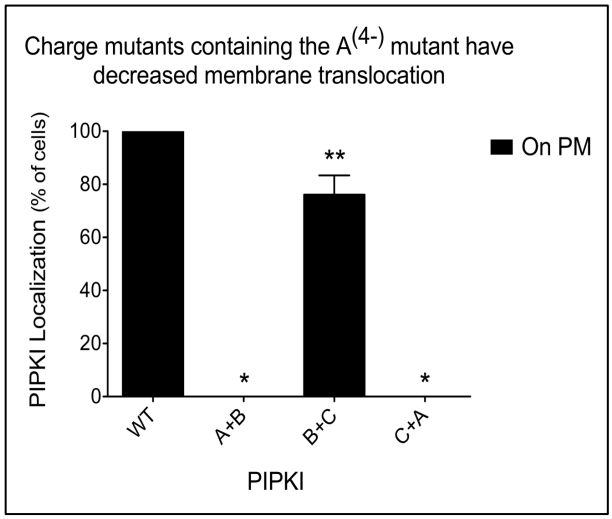


Figure 2.3B: Grouped mutants containing the A mutant show reduced plasma membrane (PM) localization. Confocal images of Cos7 cells transfected with GFP-PIPKIγ grouped mutant constructs. All PIPKIγ grouped mutants cause a significant decrease in PM localization, except the B+C mutant which still has some membrane translocation; qualitative quantification below (50 cells counted each, N=3). F-actin was stained using rhodamine-phalloidin. Arrows point to membrane localized protein. T-test *p<0.01, **p<0.05.

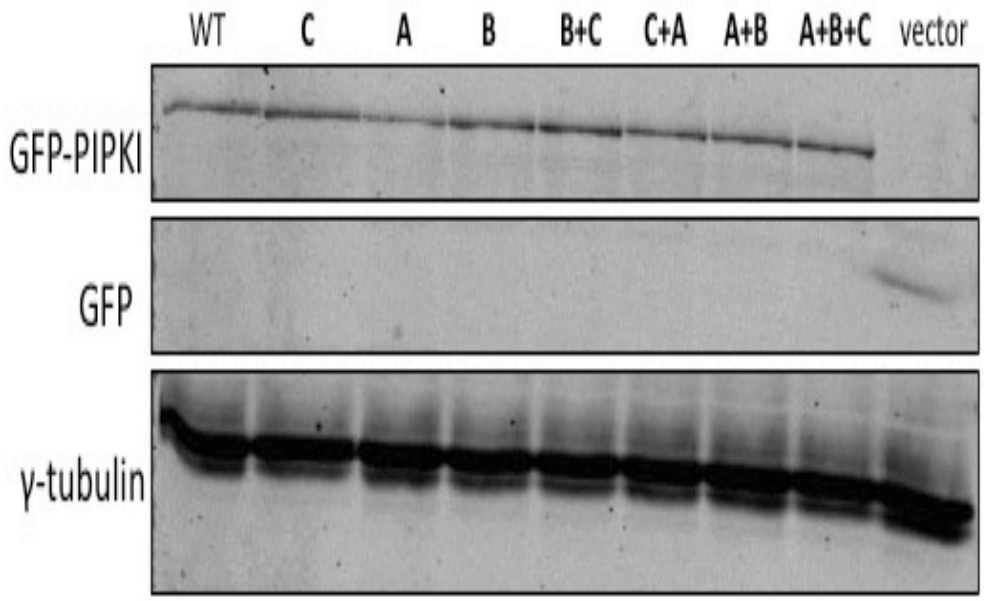


Figure 2.3C: PIPKI mutants are expressed at comparable levels. Western blot analysis of samples taken from whole cell lysates of GFP-transfected Cos7 cells using PEI for 18 hours.

PIPKIγ is known to localize to the plasma membrane. I show here that the KRHH residues play a role in this translocation perhaps by binding directly to the acidic phospholipid PA. Next, I wanted to examine whether the plasma membrane localization of PIPKIγ was dependent on membrane PA concentration or overall membrane environment. To test this hypothesis an inducible 3T3 cell line was used that overexpresses mitoPLD, mitochondria-associated PLD [63], and in doing so, produces large amounts of PA at the surface of the mitochondria (Figure 2.4A). 3T3 mitoPLD cells were transfected with GFP-tagged constructs of WT-PIPKIγ and the charge mutants. As evident from the confocal images in Figure 2.4B, PIPKIγ is not translocated to the mitochondria even when PA is

overproduced there. The charge mutants also do not localize to the PA-rich mitochondria.

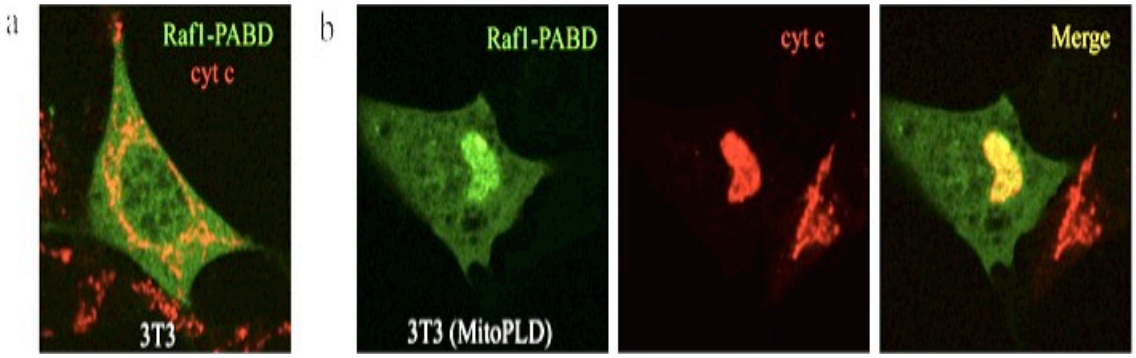


Figure 2.4A: PA is produced at the mitochondria in MitoPLD-inducible cells. NIH 3T3 cells **(a)** or 3T3 mitoPLD-inducible cells **(b)** were transfected with the PA-binding domain of Raf1 (GFP-Raf1-PABD) and stained with a cytochrome c marker for the mitochondria (unpublished results courtesy of Huiyan Huang).

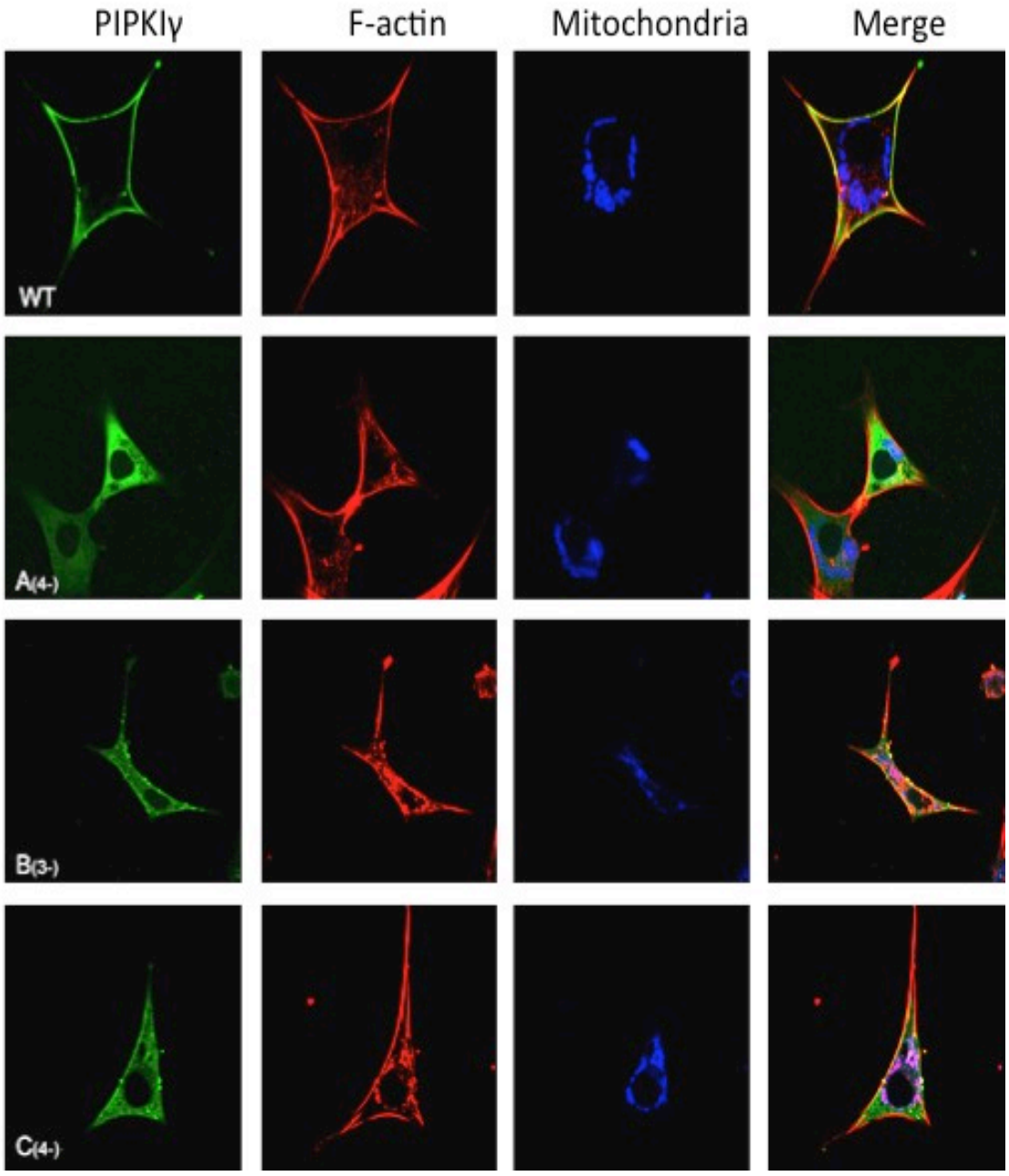


Figure 2.4B: PIPKIγ is not recruited to PA-rich mitochondria surface. Confocal images of 3T3-mitoPLD inducible cells transfected with GFP-PIPKIγ candidate site mutant constructs. PIPKIγ does not translocate to PA-rich microdomains of the mitochondria. This suggests PIPKIγ can discriminate between the two membranes, perhaps by membrane curvature, or that there are other proteins at the plasma membrane that help stabilize PIPKIγ there. F-actin was stained using rhodamine-phalloidin. Mitochondria were visualized using Mitotracker Deep Red 633.

Discussion

I demonstrate here that the KRHH residues, mutated to alanines in the A mutant, are involved in PIPKIγ plasma membrane translocation. Although the majority of lipids found at the plasma membrane are neutral, the substrate and stimulating lipids for PIPKIγ are anionic. This would suggest that the large basic area of the proposed membrane binding region would be enough to electrostatically recruit PIPKIγ to the membrane. I show here that the plasma membrane localization of PIPKIγ is not determined by overall charge, rather it depends on specific amino acid residues. The mutants generated decreased the overall charge of the proposed membrane binding region by three, four, seven, or eight and an effect was only seen when the specific KRHH residues were mutated regardless of overall charge.

The slightly decreased membrane localization of the **C** mutant could suggest that although charge is not the determining factor for PIPKIγ membrane localization, it may stabilize the protein at the membrane. This may also be the reason the **B+C** mutant had reduced membrane localization. Since the **B** mutant seems to have no effect on translocation, it makes sense that the results are the same as that observed for the **C** mutant alone. Because there is still some membrane association with the **B+C** mutant, which has a charge deficit of -7, this also implies that charge alone is not responsible for membrane recruitment. Charge may be important for initial membrane recruitment where the KRHH residues are further required to stabilize PIPKIγ at the membrane.

PIPKIγ is localized to the plasma membrane and plasma membrane-derived vesicles. I show here that the membrane localization is specific to the plasma membrane or its derived vesicles. When PA is increased at the mitochondria, PIPKIγ is not recruited to this organelle. Although this does not prove PIPKIγ is recruited to the plasma membrane by a chaperone protein or direct PA-binding, it does show that PIPKIγ localization is membrane-specific. I later provide evidence that suggests PIPKI is not chaperoned to the membrane. The plasma membrane and the mitochondria outer membrane differ by lipid composition and curvature, among other factors. PIPKIγ may use these elements to distinguish the plasma membrane from other cellular membranes. Others also suggest that the acyl chains of PA and other lipids differ depending on the membrane on which they are found. Differences in the fatty acid chain can lead to unique membrane fluidity and packing that may offer another method of membrane recognition for PIPKIγ.

The data presented here suggests that the KRHH residues may be involved in the binding of specific membrane lipids, perhaps PA, which would explain why PIPKIγ is stimulated by PA whereas PIPKII, lacking these residues, is not. I later show in Chapter 5 that these residues are in fact involved in direct binding to PA. These data are first to demonstrate specific residues in PIPKIγ that are involved in plasma membrane translocation in the absence of chaperone proteins.

CHAPTER 3: MUTATION OF THE KRHH RESIDUES CAUSES A NEAR ABOLISHMENT OF PIPKI-INDUCED ACTIN REORGANIZATION

The actin cytoskeleton is very dynamic; it is continuously undergoing changes to meet the various needs of the cell. Actin reorganization is important for many cellular processes and is regulated by many proteins. A key regulator of actin cytoskeleton reorganization is PIP2. PIP2 regulates the recruitment and activity of many of these actin organizers [54]. Increased local density of PIP2 at the plasma membrane has been shown to upregulate actin polymerization by recruiting and activating N-WASP, a key protein in filament branching [54-57]. Because PIPKIγ membrane recruitment and PA-stimulation can greatly increase PIP2 production, the role of PIPKI in actin reorganization has been widely studied [21, 23, 60, 61]. PIPKI activation can result in increased PIP2 concentration at the plasma membrane. This increase in local PIP2 density can then recruit actin remodelers like the N-WASP/Arp2/3 complex. In this chapter, I show that the four residues in the proposed membrane binding region that I have shown to be involved in plasma membrane translocation of PIPKIγ are also required for PIP2-mediated actin reorganization.

Materials and Methods

Cos7 cell culture, transfection, and staining As described in Chapter 2: Materials and Methods.

Construction of PM-tagged GFP constructs The pEGFP-PIPKIγ-**A** vector, generated as described in Chapter 2, was cut with restriction enzymes NdeI and AgeI (NEB Buffer 1 + BSA; New England Biolabs). The plasma membrane localization signal from the tyrosine kinase, Lyn (MGCIKSKRKD), was amplified from pPM-mcherry using primers CMV-F-NdeI and PM-R-AgeI (see Table 2). The PCR product was cut with NdeI and AgeI (NEB Buffer 1 + BSA; New England Biolabs). After DNA ligation, the construct (pEGFP-PM-PIPKIγ-**A)** was transformed into XL1-Gold chemically competent cells. The PM-PIPKIγ construct was generated by cutting the resultant PM- PIPKIγ-**A** vector and the pEGFP-PIPKIγ vector with restriction enzymes NdeI and BsRGI (NEB Buffer 4 + BSA; New England Biolabs). After separation on a DNA agarose gel, the pEGFP vector fragment (about 5 kilobases) and the PM-PIPKIγ-**A** insert fragment (about 1 kilobase) were subjected to DNA ligation. After ligation, the mutant constructs were transformed into XL1-Gold chemically competent cells. Generated constructs were verified by DNA sequencing using sequencing primers 5'-GATTACTTGTACTCCCTGTGC-3' and 5'-CCGTGTTGCTCATGAAC-3'.

Results

To test whether the KRHH residues, which seem to be involved in membrane translocation, have roles in actin reorganization, Cos7 cells were

transfected with GFP constructs of the charge mutants. An easy and reliable measure of actin reorganization by PIPKI, as identified by Rozelle, et al, is the formation of actin comets and foci [64]. Comets are formed in response to overexpression of PIPKI and usually carry cargo in their comet heads (the authors did not fully characterize actin foci). Actin comets are defined as filamentous actin-containing structures that resemble the comets naturally found in Xenopus oocytes, and adipocytes, among others, and cells infected with *Listeria* or *Shigella* bacteria [65-67](Figure 3.1A). Here, actin foci are defined as smaller, punctate or doughnut-shaped structures also containing filamentous actin. Each transfected and stained cell population was analyzed for actin comet and foci formation (non-transfected cells do not have significantly visible actin comets or foci) using the Leica Confocal Microscope. Comets and foci were counted, separately, using a minimum of 20 transfected cells for each construct (N=3). Statistical relevance was determined using the t-test compared to WT values.

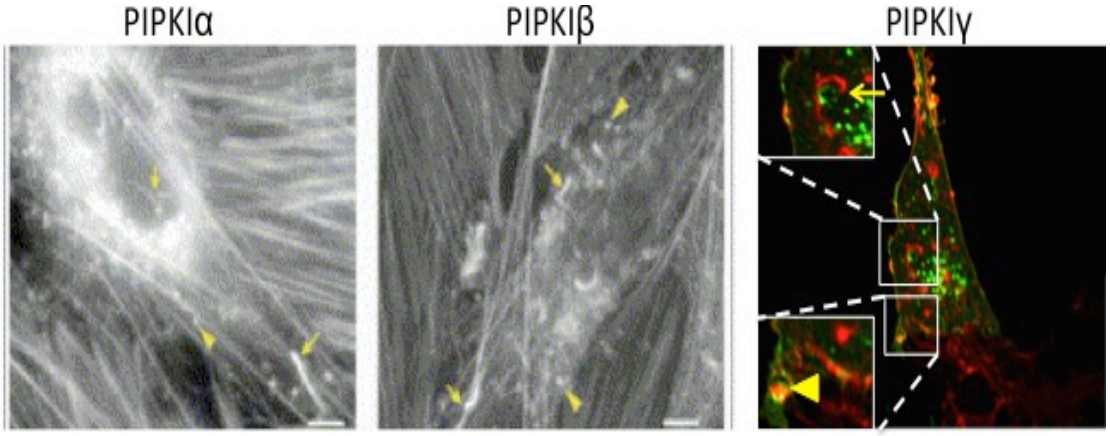


Figure 3.1A: Characterization of actin comets and foci. Phalloidin staining of REF52 cells overexpressing PIP5KIα and PIP5KIβ (Rozelle, et al. Current Biology, 2000), and Cos7 cells overexpressing PIPKIγ. Arrows point to actin comets and arrowheads point to actin foci.

Of the transfected cells, those transfected with WT-PIPKIγ or the **B** or **C** mutants all produced comparable numbers of actin comets and foci (Figure 3.1B,D), suggesting that the corresponding amino acid residues have no direct involvement in actin reorganization. The **A** mutant transfected cells, however, had a dramatic decrease in actin comet/foci production. To ensure that this was a direct result of the KRHH residues and not a charge-dependent phenomenon, Cos7 cells were also transfected with the grouped mutants. The charge mutants containing the **A** mutant showed a significant decrease in comet and foci formation whereas the grouped mutants without the **A** mutant behaved as WT-PIPKIγ (Figure 3.1C,D). These data suggests that the KRHH residues are involved in and are important for actin reorganization.

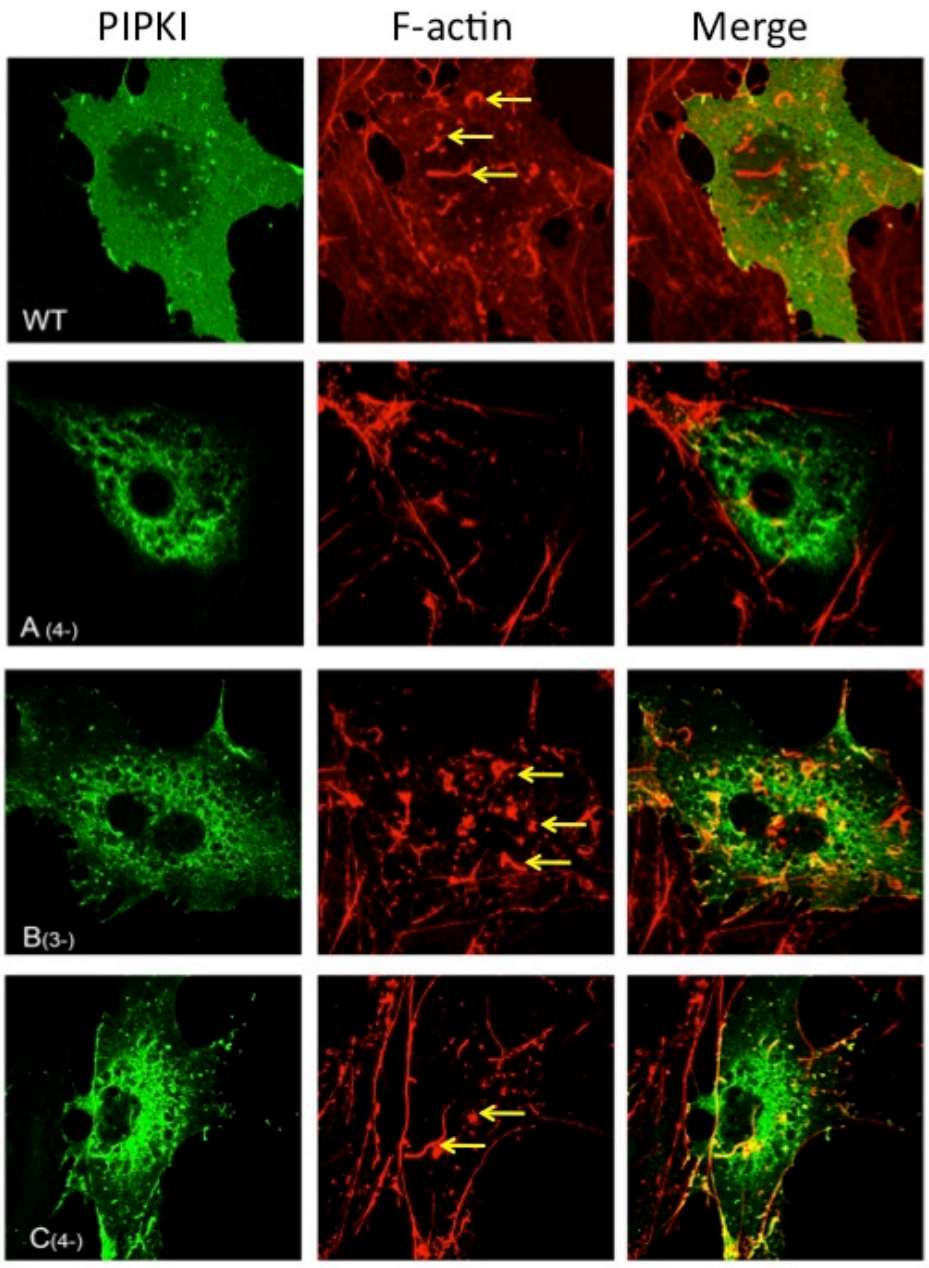


Figure 3.1B: Actin reorganization induced by PIPKIγ is dependent on residues mutated in A mutant. Confocal images of Cos7 cells transfected with respective pEGFP-PIPKIγ (green) candidate site mutant constructs. Cells were fixed then stained with rhodamine-phalloidin (red) and the number of actin foci and actin comets for 20 cells in each population were counted. Arrows point to comets/foci.

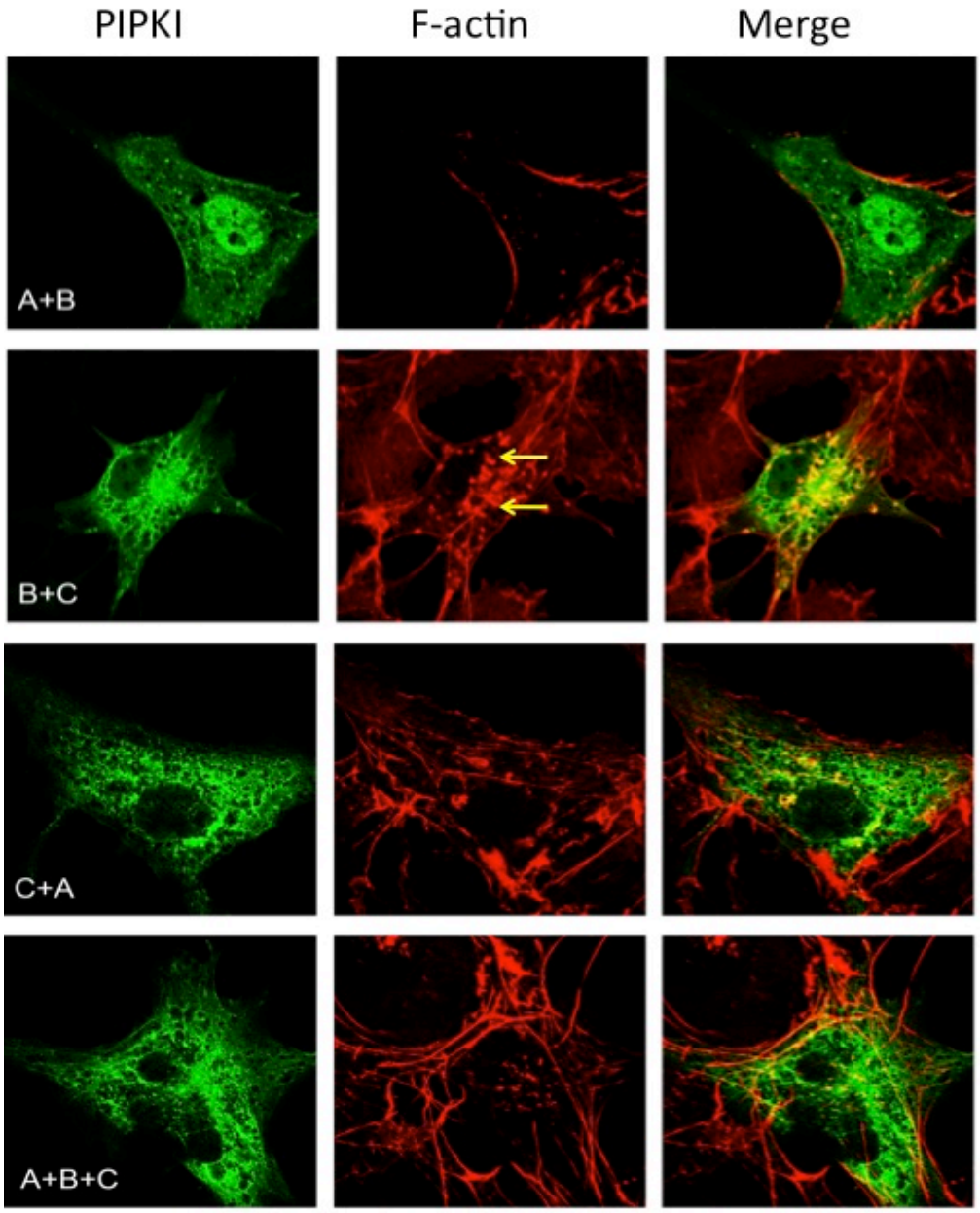


Figure 3.1C: Actin reorganization induced by PIPKIγ is dependent on residues mutated in A mutant and not by charge alone. Confocal images of Cos7 cells transfected with respective pEGFP-PIPKIγ (green) charge mutant constructs. Cells were fixed then stained with rhodamine-phalloidin (red) and the number of actin foci and actin comets for 20 cells in each population were counted. Arrows point to comets/foci.

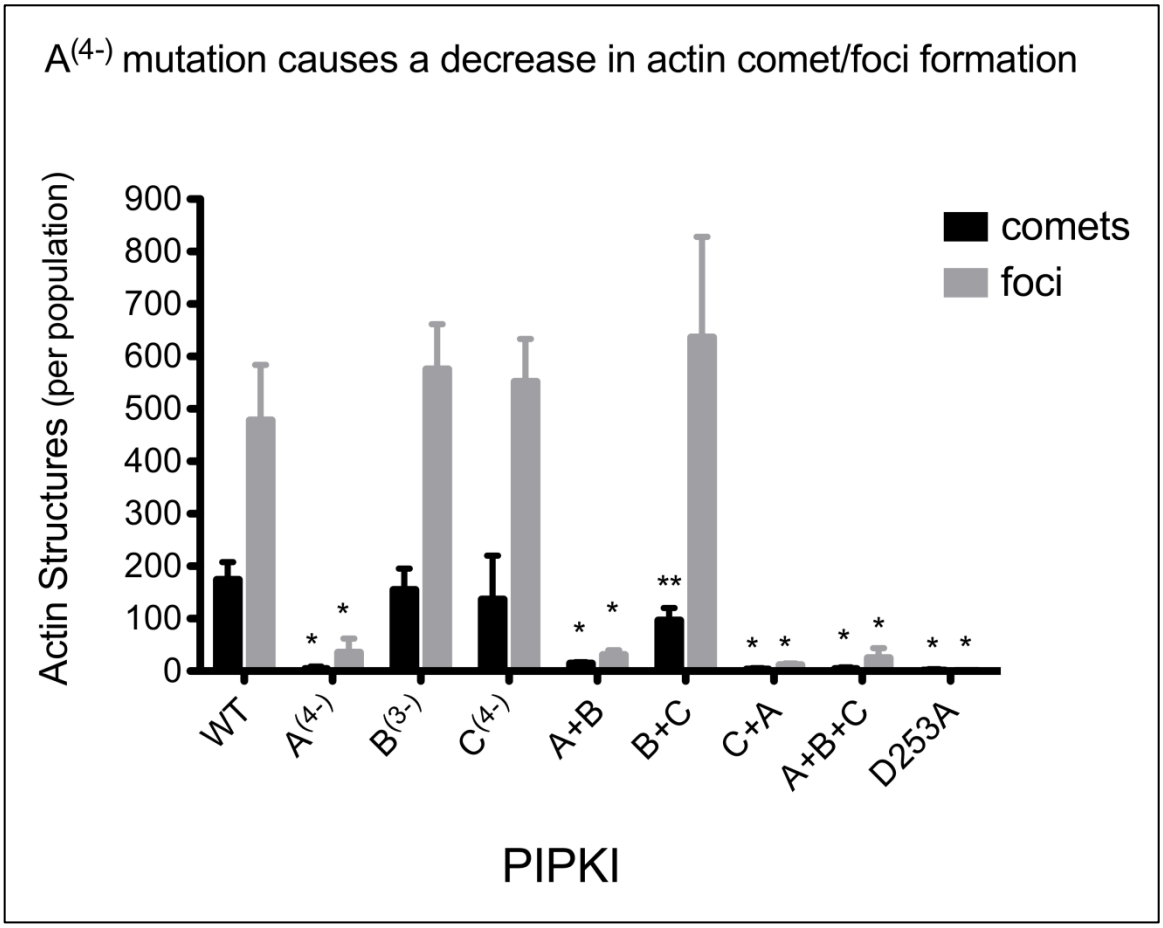


Figure 3.1D: The KRHH residues, mutated in the A mutant, are involved in PIPKIγ-mediated actin reorganization based on the formation of actin comets and foci in overexpressed cells. Graphical representation of total number of actin structures formed in 20 cells within each population, over three experiments. T-test *p<0.01, **p<0.05. D253A is the PIPKI kinase dead protein, which expectantly does not induce actin reorganization.

The inability of the **A** mutant to induce actin comet formation could be a direct result of insufficient membrane translocation. To test this possibility, we made a plasma membrane-targeted construct of the **A** mutant (denoted PM-**A**) and transfected it into Cos7 cells (Figure 3.2). As expected, the PM-WT control construct (wild-type PIPKIγ containing the plasma membrane targeting signal) localized to the plasma membrane and was able to induce actin comets similar to

the original WT construct. The original **A** mutant does not localize to the plasma membrane. Interestingly, the PM-PIPKIγ-**A** construct, although it was able to translocate to the plasma membrane, did not induce actin reorganization and could not form actin comets. This suggests that membrane translocation alone is not sufficient to induce actin reorganization and that these four residues, specifically, play some role in PIPKI-mediated actin reorganization.

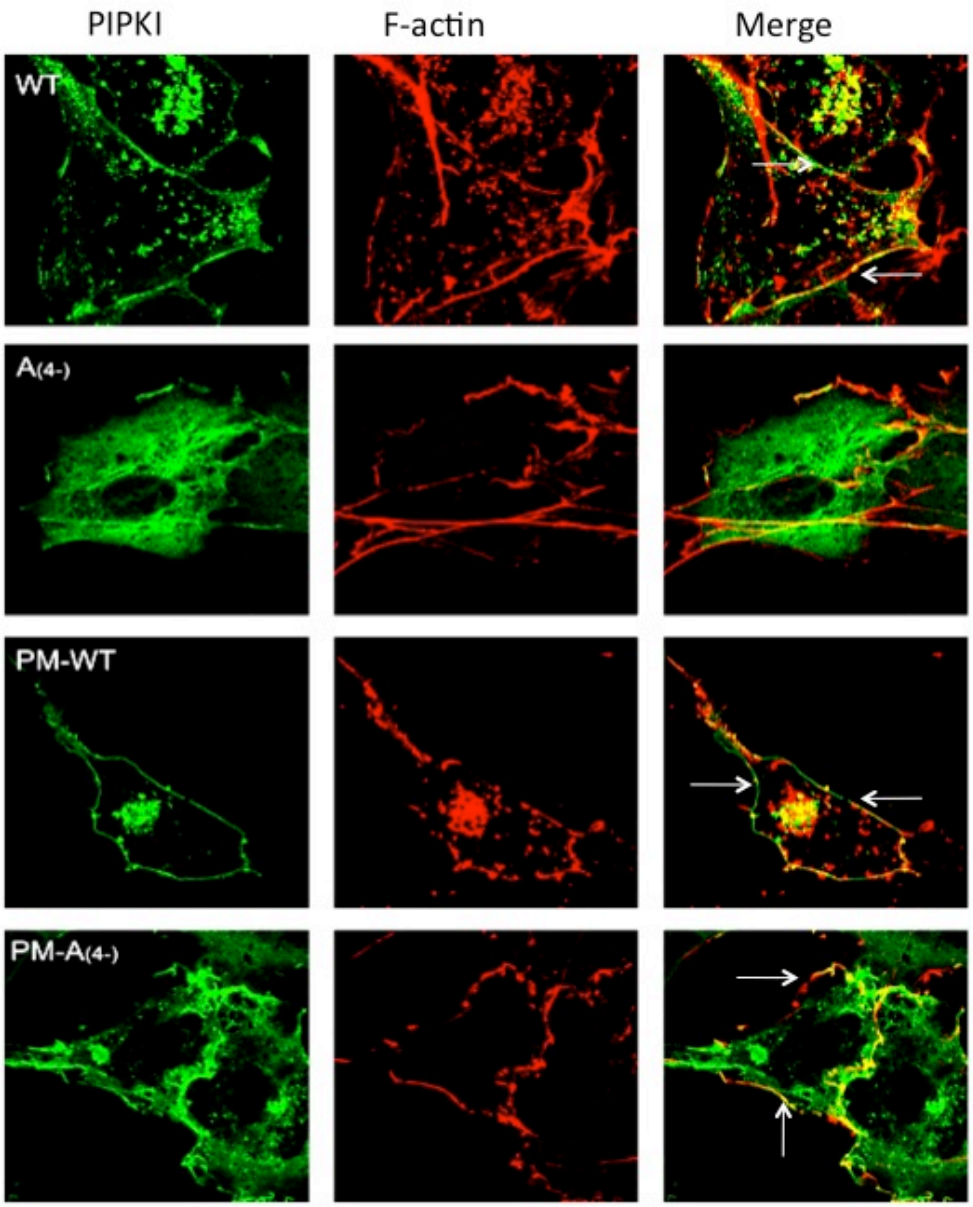


Figure 3.2: Targeting the A mutant to the plasma membrane alone cannot induce actin comet formation. Cos7 cells transfected with GFP constructs with an N-terminal fused plasma membrane binding signal (from Lyn). This suggests that membrane recruitment alone is not sufficient for actin reorganization. Chaperones alone cannot account for this phenotype; perhaps PA-binding is also needed. F-actin was stained using rhodamine-phalloidin. Arrows point to membrane localized protein.

Discussion

PIPKIγ has a substantial role in actin reorganization because of its ability to increase the local density of PIP2 at the plasma membrane. I have identified four residues in PIPKIγ that when mutated abolish actin polymerization. The KRHH residues are important for actin comet and foci formation, a direct measure of PIP2-mediated actin reorganization. This effect is specifically due to these four residues and is not due to charge. The **C** mutant had a similar charge deficit to that of the **A** mutant but did not effect actin comet formation. To confirm this hypothesis the grouped mutants, possessing a greater charge deficit, were also tested.

The grouped mutants were unable to form actin comets except for the **B+C** mutant, which behaved like WT. This is in line with the charge mutant data to confirm that the KRHH residues are involved in actin reorganization since the other grouped mutants that were unable to form comets all possessed the **A** (KRHH) mutant.

I cannot rule out the possibility that the KRHH residues directly bind and recruit an actin remodeling protein from this data alone. If this were the case, mutating these residues would generate the same results. To date, however, no known PIPKI-binding protein has a binding region that includes these residues.

Taken together, the data presented in this chapter suggest that although membrane translocation is important for PIPKI-mediated actin reorganization, it alone is not sufficient. PIPKIγ must also maintain the ability to be stimulated by

PA. This may produce the high local density of PIP_2 needed for the recruitment and regulation of actin remodeling proteins at the plasma membrane. I show in the following chapter that the KRHH residues in the proposed membrane binding region of PIPKIγ are directly involved in PA-stimulation. The data presented here show how these residues effect PIPKIγ function. The next few chapters investigate the mechanistic role by which KRHH may regulate these functions.

CHAPTER 4: THE KRHH RESIDUES ARE NECESSARY FOR PA STIMULATION OF PIPKIγ

PIPKI is the main enzyme responsible for PIP_2 generation at the plasma membrane. PIP_2, in turn, is necessary for the recruitment of actin remodeling proteins, such as N-WASP and talin [11, 54, 57]. At basal levels, PIPKIγ does not produce enough PIP_2 to generate actin structures such as actin comets and foci, or PIP_2 may be masked by other proteins at the membrane. Large amounts of PIP_2, produced by stimulating PIPKI activity with PA or small GTPases, are needed for such structures. Following the functional data presented above, I show here that the KRHH residues in the proposed membrane binding region of PIPKIγ are necessary for PA stimulation of PIPKIγ, offering a mechanistic role for these four residues. The kinase activity of PIPKIγ mutants was measured to determine whether the mutated residues were necessary for PA stimulation.

The kinase domain of the PIPKI protein is large; it comprises nearly 50% of the total protein or about 330 amino acids. Mutating a few amino acids as I have done here should not greatly affect the overall protein structure, as supported by the similar expression level in mammalian cells (see Figure 2.3C). The substrate binding site is regulated by a glutamate residue at position 410 [3, 4], more than 200 amino acids from the nearest residue mutated. So, again, mutating residues at the proposed membrane binding region should not and

does not affect basal kinase activity or substrate binding. The goal is to determine whether the KRHH residues that seem to have a role in PIPKIγ membrane translocation and actin reorganization are involved in PA stimulation of PIPKIγ kinase activity. If it is, the **A** mutant should not be able to produce high levels of PIP_2 in the presence of PA.

To best model a lipid bilayer in our kinase assays, synthetic liposomes were used as a lipid source for the assays. Uniform, bilayer liposomes contained phosphatidylcholine (PC), substrate phosphatidylinositol 4-phosphate (PI(4)P), with or without stimulant PA.

Materials and Methods

Construction of pET24 constructs The pEGFP-WT-PIPKIγ and mutant vectors, generated as described in Chapter 2, and pET24 vector was cut with restriction enzymes EcoRI and HindIII (NEB Buffer 2 + BSA; New England Biolabs). After separation on a DNA agarose gel, the pET24 vector fragment (about 5 kilobases) and the pEGFP insert fragments (about 1 kilobase) were subjected to DNA ligation. After ligation, the mutant constructs were transformed into XL1-Gold chemically competent cells. Generated constructs were verified by DNA sequencing using sequencing primers 5'-GATTACTTGTACTCCCTGTGC-3' and 5'-CCGTGTTGCTCATGAAC-3'.

Expression and Purification of Recombinant PIPKIγ proteins Recombinant WT and mutant PIPKIγ were expressed in *E. coli* BL21(λDE3) or Rosetta 2 chemically competent cells with an N-terminal T7 tag and C-terminal His-tag and purified by Ni^{2+}-chelate chromatography according to the manufacturer's protocol (Qiagen). Proteins were then dialyzed against 1) 20mM Tris, pH 7.6, 200mM NaCl, 5mM β-mercaptoethanol and then 2) 20mM Tris, pH 7.6, 100mM NaCl, 5mM β-mercaptoethanol to exchange the buffer and remove the imidazole used in purification. The proteins were collected, aliquoted, flash-frozen in liquid nitrogen, and stored at -80^{o}C. I found that PIPKI proteins are not very stable even at -80^{o}C; proteins purified 3 months prior, though stored at -80^{o}C, were unable to produce even basal level activity. When purifying proteins, WT PIPKI and all the mutants were purified within two days of each other and used as soon as possible. The fact that the batch purified proteins all maintained comparable levels of basal activity allow us to compare them to each other, but only in this case.

Liposome Preparation POPC (phosphatidylcholine), POPA (phosphatidic acid), and Brain PI(4)P were purchased from Avanti Polar Lipids. Large unilamellar vesicles (LUV) were generated by mixing POPC with PI(4)P (80:20) or POPC and PI(4)P with POPA (60:20:20) dissolved in chloroform in the designated ratios. Mixed lipids were dried in a round bottom flask under rotary evaporation placed under vacuum for 30 min then resuspended in 176 mM Sucrose, 20 mM Tris (pH 7.6) at 2 mM. Hydrated lipids were subjected to at least six cycles of

freeze thawing in liquid nitrogen/ 37^{o}C water bath before ten cycles of extrusion through a 100nm membrane filter with a lipid extruder (Northern Lipids). Liposome size was verified by electron microscopy. These 100nm sucrose-loaded liposomes (now at approx. 1.3 mM) were collected after ultra-centrifugation at 100,000*xg* then resuspended in 20 mM Tris (pH 7.6), 100mM NaCl. Liposomes were composed of 400uM total lipids, 200uM were accessible to the protein (half in the inner layer, half in the outer layer). The exposed PC/PI/PA lipids were at a ratio of 60/20/20: 120uM PC, 40uM PI(4)P, and 40uM PA. The exposed PC/PI lipids were at a ratio of 80/20: 160uM PC, 40uM PI(4)P.

PIPKI-Liposome Kinase Activity Assay Activity assays were performed for 15 min at room temperature in 100ul reactions containing 100ng purified protein, 400uM prepared liposome, 20mM Tris (pH 7.6), 100mM NaCl, 50uM ATP, 10mM $MgCl_2$, and 5uCi [^{32}P]-γATP/reaction. Reactions were terminated using 1M HCl, the lipid products were extracted with 1:1 chloroform:methanol, washed with 1:1 1M HCl:methanol, and separated on a Silica Gel H/ 1% potassium oxalate-pretreated TLC plate (Analtech, Inc) developed in chloroform:methanol:ammonium hydroxide:water (90:90:13:9). The TLC plate was exposed to autoradiography film overnight. The corresponding product bands from the film were scrapped from the TLC plate into scintillation vials, dissolved in EcoLume counting liquid, and quantified on a Beckman Coulter LS 6500 Liquid Scintillation Counter. PIP2 produced, reported as fmol per ng protein, was calculated using the equation (cpm/fmol)= (Ci/mmol) x 2.22 x E to calculate fmol (cpm is counts per minute

from the scintillation counter, Ci/mmol is the specific radioactivity on the day of use, and E is the counter efficiency). This value was then divided by the amount of protein (ng) used in the assay. T-test, activity without PA compared to activity with PA, was used to determine statistical relevance.

Results

The bacterial PIPKI expression constructs used in previous literatures contained an N-terminal tag for identification and/or purification. However, the yield of full-length proteins from these constructs were very low. In order to obtain the larger amounts of protein needed for my biochemical studies, different expression vectors were tested adding varying tags in order to identify one type of construct that yielded fair amounts of pure protein. Some N-terminal tags (his, T7, intein; from pET and pTXBI vectors) produced truncated protein products, verified by western blot analysis, even in the presence of protease and phosphatase inhibitors (Figure 4.1). I tried to further separate the full-length protein from these truncated proteins by ion exchange chromatography but was unable to obtain a level of separation that would allow isolation of the full-length protein alone. The truncation could be due to protein cleavage or incomplete translation. I made a PIPKI construct with a T7 N-terminal tag and a C-terminal His tag. The logic here being that during purification using his tag, only full-length protein would bind to the Ni-NTA agarose beads thereby reducing or eliminating

the amount of truncated proteins bound. Also, adding the N-terminal T7 tag would allow further purification if needed (Figure 4.1).

Two E.coli competent cell strains, Rosetta 2 and BL21(DE3), were tested to identify which produced less truncated proteins. Proteins expressed in the Rosetta cells were full-length with less truncated products (Figure 4.1). Rosetta cells have additional tRNAs used in mammal cells that may aide in producing more full-length proteins (Novagen Competent Cells Brochure[68]).

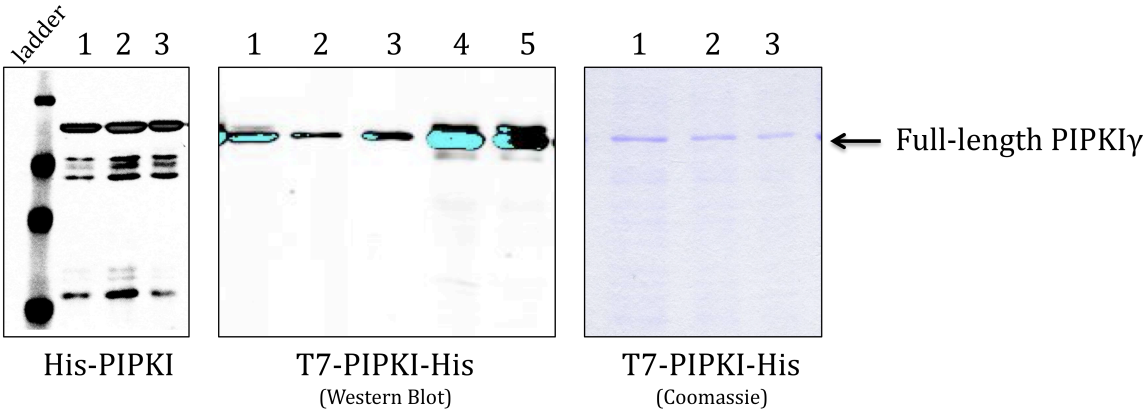


Figure 4.1: The C-terminally fused His-tag allows the purification of the full-length WT-PIPKIγ protein. Left panel: Western Blot of elution fractions from Ni-NTA purification of a His-PIPKI protein construct. Multiple bands demonstrate truncated proteins are not removed during purification. Lane 1: elution fraction 1, lane 2: elution 2, lane 3: elution 4. Middle panel: Western Blot of elution fractions from Ni-NTA purification of a T7-PIPKI-His protein construct. Single band demonstrate truncated proteins are removed during this purification process, and are further purified by dialysis. Lane 1: flow through, lane 2: wash, lane 3, elution 1, Lane 4: elution fraction 2, lane 5: elution 3. Right panel: Coomassie Blue-stained SDS protein gel of elution fractions from Ni-NTA purification of a T7-PIPKI-His protein construct expressed in Rosetta 2 cells. Lane 1: elution fraction 1, lane 2: elution 2, lane 3: elution 3.

To investigate whether the KRHH residues in PIPKIγ (mutated in the **A** mutant) are involved in PA stimulation, PIP_2 production was measured in the presence or absence of PA in a standard *in vitro* kinase assay. After separation by thin layer chromatography, the PIP_2 product was collected and counted for

WT and the **A** mutant. The TLC developing solution allows for the separation of PIP2 from unincorporated lipids as well as other lipids in the liposomes (Figure 4.2A, [69, 70]). To confirm this, a TLC plate was spotted with cold, unlabeled PIP2 and a kinase assay reaction with WT-PIPKI. The final developed TLC plate was further developed in a chromatography developing chamber with a few iodine crystals to visualize the cold PIP2 to ensure that the lipid produced in our kinase assay was indeed PIP2 (data not shown).

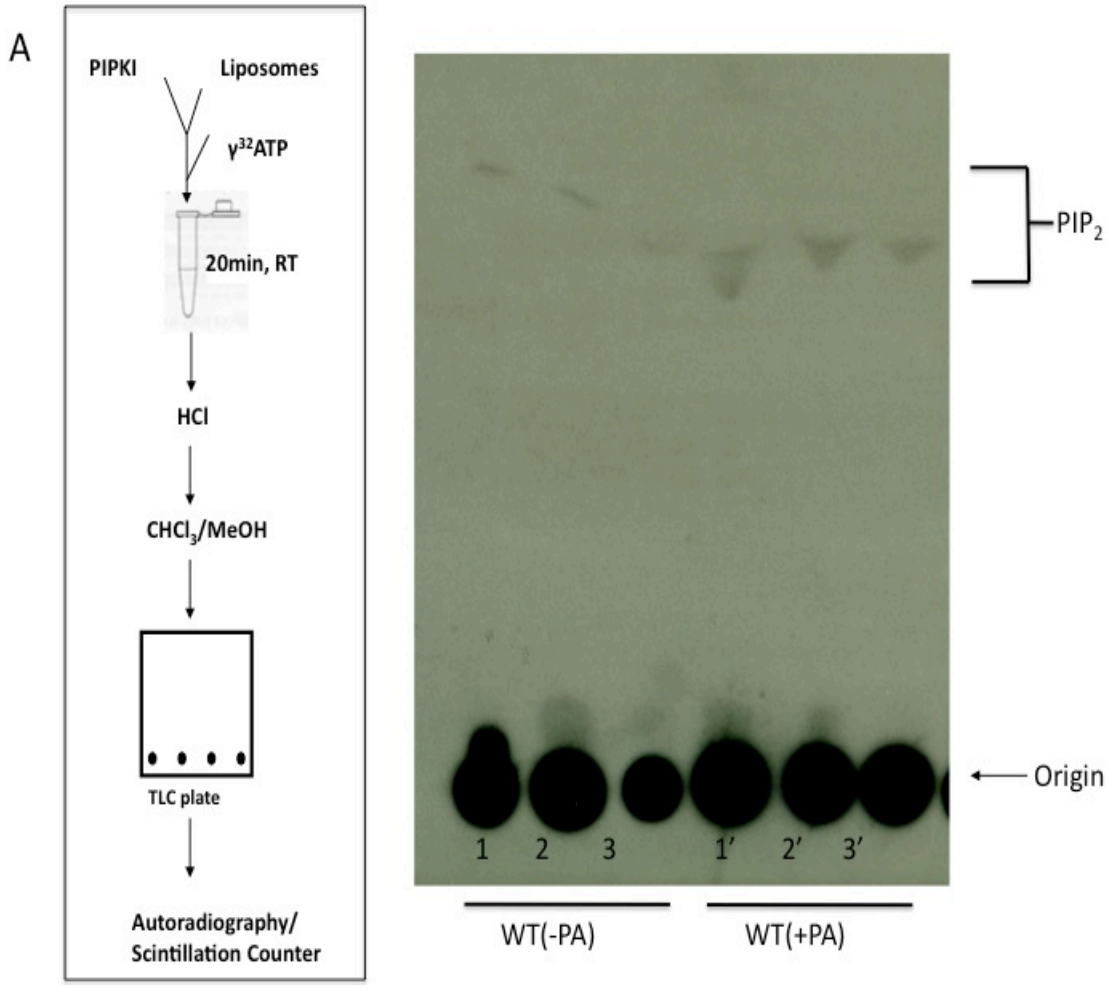
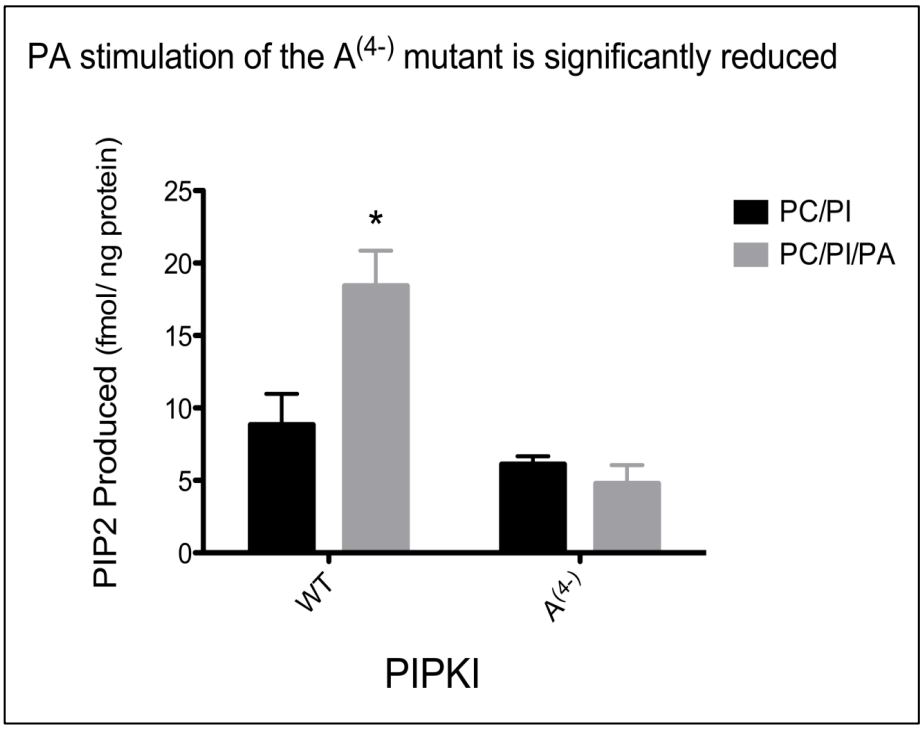


Figure 4.2A: The kinase activity assay is a reliable measure of PIP_2 produced by PIPKIγ. The left panel is a representative diagram of the kinase activity assay performed on PIPKIγ purified proteins. The representative autoradiography film of the assay with WT protein shows the results of three experiments. PIP2 band location can differ depending on the running pattern of the solvent front.

From the kinase assay, it is clear that both the WT and A mutant proteins maintained basal activity, suggesting that they were both indeed intact and functional (Figure 4.2B). Whereas the kinase activity of the WT protein was stimulated by PA, the **A** mutant was not stimulated.

To further analyze the residues that may be involved in PA stimulation, the **A** (KRHH) mutant was broken down into its double mutant components: K97A/R100A and H126A/H127A. The activity of these double mutants was measured in the presence and absence of PA. Interestingly, although the **A** mutant was not stimulated by PA, the activity of its component double mutants were both PA-stimulated (Figure 4.2B).



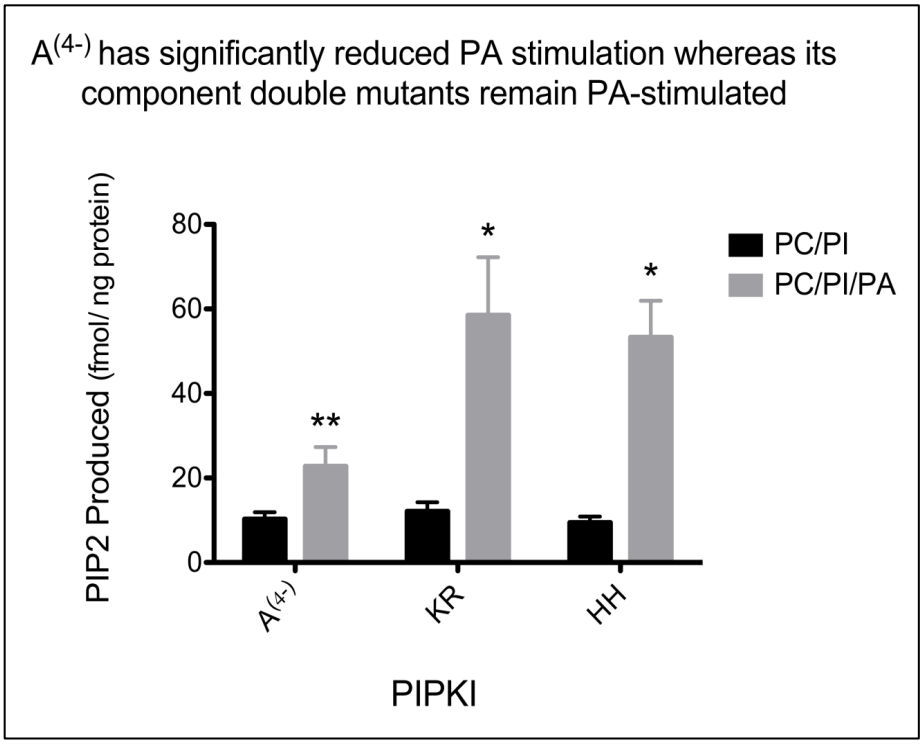


Figure 4.2B: The kinase activity for the A mutant is not stimulated by PA. The graphs show data of the kinase activity of PIPKIγ proteins in the presence of PI(4)P substrate [PI], with or without phosphatidic acid [PA]. Top: Although the A(4-) mutant maintains basal activity, it is not stimulated by PA. Bottom: The double mutant components of A(4-), KR/AA and HH/AA, are still stimulated by PA and do not account for the inability of A(4-) to be stimulated by PA. T-test, *$p<0.01$, **$p<0.05$.

Discussion

Using a standard PIPKI kinase assay, I show that the residues KRHH are necessary for PA stimulation of PIPKIγ. These residues, mutated in the **A** mutant, produce only basal levels of PIP_2 in the presence of PA. Since these residues are involved in targeting PIPKIγ to the plasma membrane where it is stimulated by the membrane lipid, PA, to produce high amounts of PIP_2 necessary for actin reorganization, these results also suggests that these residues may be involved in PA-binding.

In order to identify the minimal region of PIPKIγ necessary for stimulation by PA, the **A** mutant was deconstructed into its double mutant components, which were found to behave like WT. This also suggests that all four residues together, K97, R100, H126 and H127, are involved in PA-stimulation of PIPKIγ.

The *in vitro* nature of the assay implies that chaperones are not needed to recruit PIPKIγ to the membrane lipids or for stimulation by PA because only purified PIPKIγ, liposomes, ATP, and magnesium chloride are included; there are no interfering proteins or chaperones available in the system. However, if the overall membrane environment of the plasma membrane offers some selectivity in PIPKIγ recruitment, how does this oversimplified assay work as it has for many others? One explanation lies with Brownian protein diffusion/collision dynamics. The K_m (Michaelis constant) of PIPKIγ for PI(4)P in the absence of PA as reported by Ishihara, et al is 37uM. Because protein activity is very fast, PIP_2 can be produced rapidly at the membrane surface when PIPKIγ collides with the PA-

and PI(4)P-rich liposomes. This observation has previously been described for many enzymes [71-74]. These discrepancies demonstrate that regulation of PIPKIγ is more complex than originally understood.

CHAPTER 5: THE KRHH RESIDUES ARE INVOLVED IN DIRECT BINDING TO PA

Few proteins that are regulated by PA have had their "PA-binding regions" mapped. Of these, the majority have been narrowed down to a handful of residues that when mutated or deleted result in decreased PA-binding (see Table 1). The identified PA-binding regions are usually rich in basic amino acids, suggesting that these positively charged sites in the protein bind electrostatically to the negatively charged PA lipid.

Electrostatic binding, however, does not fully explain how PA regulates these proteins. Phosphatidylserine and other negatively charged lipids exist at the plasma membrane, usually at higher concentration than PA. So how do these proteins distinguish between these lipids if binding is electrostatic alone?

Direct binding to PA has been shown for some PA-binding proteins. In these cases, specific residues, and not charge alone, have been shown to be directly involved in PA-binding. In this chapter, I show that the KRHH residues, which I have shown to be involved in PIPKIγ membrane translocation, actin reorganization, and stimulation by PA, directly bind PA.

Materials and Methods

Expression and Purification of Recombinant PIPKIγ proteins As described in Chapter 4: Material and Methods

Liposome Preparation As described in Chapter 4: Material and Methods

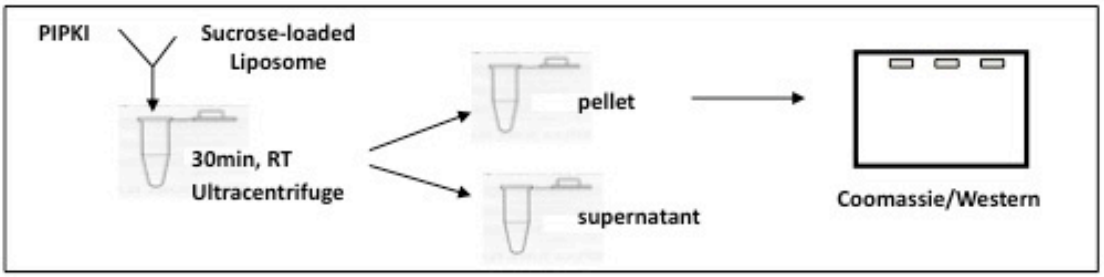
PIPKI-Liposome Binding Assay The protein was pre-cleared at 100,000xg to remove any protein aggregates before adding it to the assay. The percent of protein bound was calculated as a function of individual protein input: ([%bound$_{-PA}$] – [%bound$_{+PA}$])/input. Binding assays were performed by mixing 100ng of the pre-cleared protein and 400uM sucrose-loaded liposomes in 20 mM Tris (pH 7.6), 100 mM NaCl, 1 mM DTT, and 1mM EDTA. Reactions were incubated for 30 min at room temperature then ultra-centrifuged at 100,000*xg* for 30min. Liposome pellets were resuspended in SDS-PAGE buffer and resolved by SDS-PAGE. Western Blot analysis was performed using primary antibody mouse anti-His (Clontech) and Alexa 680 goat anti-mouse secondary antibody (Molecular Probes). Band intensity was quantified using the LI-COR Odyssey Infrared Imaging System. T-test, compared to WT values, was used to determine statistical relevance.

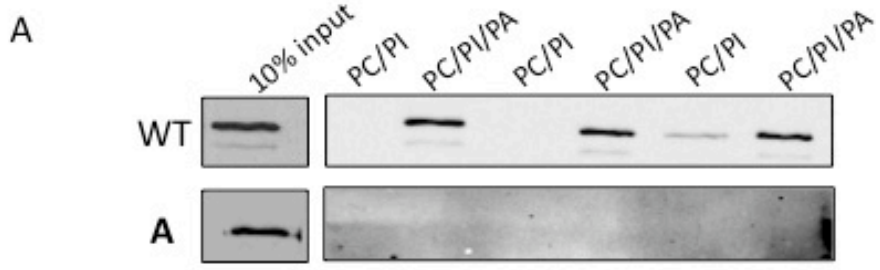
Results

To better investigate the role of these specific residues in PA regulation of PIPKIγ, an *in vitro* binding assay was used to evaluate the ability of the **A** (KRHH) mutant to bind to PA-containing liposomes. WT protein and the **A** mutant were added to sucrose-loaded liposomes with or without PA. After centrifugation

at 100,000xg, the supernatant was removed and the pellet, containing the liposomes and any bound protein, was resuspended and analyzed by Western Blot. As suggested by Jarquin-Pardo, et al. the WT protein was able to bind PA-containing liposomes, about 33% bound to the PA liposomes (Figure 5A,B). When the KRHH residues are mutated to alanines, that binding is abolished (about 1% bound). This suggests that the KRHH residues are involved in direct interaction with PA lipids.

In order to identify the minimum region of PIPKIγ involved in direct PA-binding, the double mutant components of the **A** mutant were tested for PA-binding (Figure 5B.) The binding assay of the double mutants showed reduced binding to PA. Although binding was not abolished as it was for the **A** mutant, the composite of the double mutants, it suggests that the all four residues are required for PA binding.





B

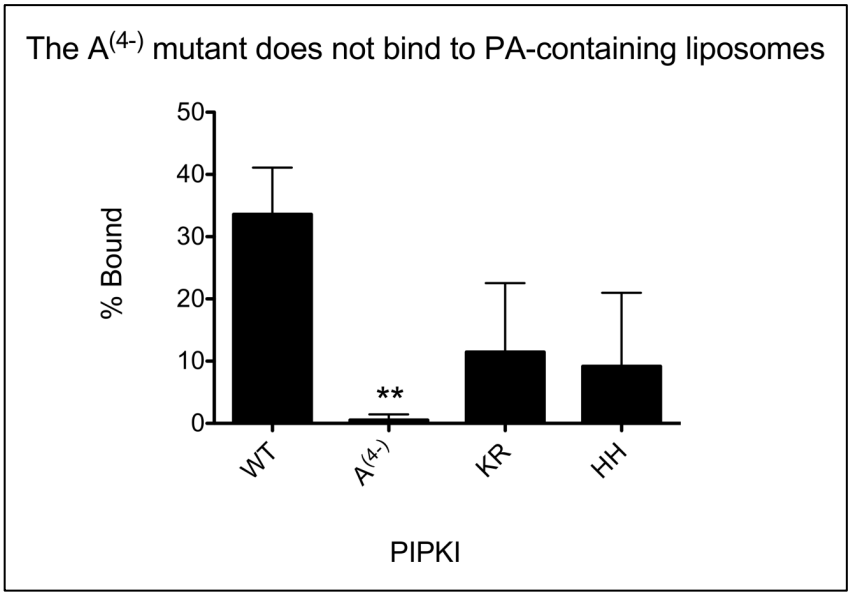


Figure 5: The A(4-) mutant does not bind PA, whereas its double mutant components show only reduced binding. Top Panel: Representative diagram of the liposome binding assay. Purified recombinant PIPKIγ wt or mutant protein was added to sucrose-loaded liposomes ± PA. The pelleted fraction was collected after centrifugation and analyzed by Western Blot. **A)** Representative Western Blot analysis of samples ± PA-containing liposomes for WT protein and the A mutant, in triplicate. **B)** Graph of assay results. %Bound is calculated as [(%bound +PA)-(%bound –PA)]/input, over three experiments. T-test compared to WT, **p<0.05.

Discussion

Data presented in the previous chapters show that the KRHH residues are involved in the regulation of PIPKIγ. Here I show that these residues are involved in direct binding to PA. When the KRHH residues are mutated, PIPKIγ no longer binds to PA-containing liposomes. In order to pinpoint the residues involved in direct PA binding, the double mutant components of the **A** mutant were tested, K97A/R100A and H126A/H127A. Although the two mutants exhibited only a slight decrease in binding, the large error bars indicate that binding of these mutants to the membrane may be unstable. This also explains why these mutants can still be stimulated by PA. Because kinase activity has a quick turnover, PIP_2 can be produced even when PIPKIγ can only bind briefly to the membrane.

Stace, et al. have previously identified a region in PIPKIβ that they suggest is involved in PA-binding [75]. The authors were unable to test their proposed PA-binding mutant using standard binding assays and instead used a less direct membrane translocation assay designed by the group. In their assay, cytosol from PIPKIβ-expressing Cos7 cells were incubated with purified Golgi-enriched membranes in the presence or absence of bacterial PLD. PA-binding of their mutants was determined by whether or not the mutant proteins bound to these membrane fraction, where the exogenous PLD should have produced PA, after centrifugation as detected by Western Blot analysis. The amino acids involved in PA-binding that they identified were just outside the proposed

membrane binding region and failed to produce basal levels of PIP_2 in the absence of PA in their kinase assays. The methods I use in this study are well established and more direct. Also, the KRHH residues I identify are within the proposed membrane binding region of PIPKIIγ and do maintain basal kinase activity (see Chapter 4).

The identification of the KRHH residues as the PA-binding region of PIPKIγ is analogous to those identified for many other PA-binding proteins (see Table 1.) These regions also have a high density of basic residues but do not possess a consensus sequence. My results suggest that the KRHH residues in PIPKIγ directly bind to PA and that this binding is required for PIPKIγ membrane translocation, stimulation by PA, and, ultimately, PIP_2-mediated actin cytoskeleton reorganization.

CHAPTER 6: CONCLUSIONS AND DISCUSSIONS

I demonstrate here that the KRHH residues in the proposed membrane-binding region of PIPKIγ are involved in actin reorganization. These residues are also important for plasma membrane translocation and PA stimulation of PIPKIγ activity. Taken together, the data presented here also suggests that although membrane translocation is independent of activity (kinase-dead PIPKIγ can still translocate to the membrane; data not shown), PA-binding, membrane translocation, and kinase activity are required for actin reorganization.

The KRHH residues in PIPKIg are involved in plasma membrane localization

PIPKI membrane recruitment is essential for its function; the substrate PI(4)P and stimulating lipid PA all reside at the membrane. Although direct lipid binding is an attractive model for PIPKI membrane targeting, other data suggests that it may not be solely responsible. Recent reports have identified other membrane-associated proteins, like talin, integrin, and WASP-Arp2/3, that interact or bind to PIPKI that may recruit PIPKI to the membrane even in the absence of PA [6, 11, 27, 64, 76]. Also PIPKI does not have a typical lipid-binding module, such as a PH domain. PA binding sites in many PA-binding proteins, such as p47, Raf, and mTOR, have been known to be mediated by polybasic amino acid regions [44, 45, 48, 50, 51].

Because all 3 isoforms of PIPKI are activated by PA, I identified a conserved pattern of basic residues present amongst the PIPKI isoforms but absent from PIPKII which is not stimulated by PA. The proposed membrane association region of PIPKIIβ provided clues as to where the membrane associated region of PIPKI may be located[35]. I show in this dissertation that the K97, R100, H126, H127 (KRHH) residues in PIPKI are involved in membrane recruitment. When these residues are mutated to alanine, membrane localization of PIPKI is abolished.

I also show that charge alone is not sufficient to recruit PIPKI to the plasma membrane. When the KRHH residues are not mutated, PIPKI is still recruited to the membrane even at a local charge deficit of -8. This demonstrates that these four residues are involved in PIPKI plasma membrane recruitment.

The data presented here also demonstrate that PIPKI is recruited to the plasma membrane but cannot be recruited to PA-rich microdomains on the mitochondria surface. When PA production is increased by the induction of a mitochondria-associated PLD on the mitochondria surface, PIPKI cannot be recruited there. PIPKI may use membrane curvature and fluidity to distinguish between the two organelles.

The KRHH residues are also involved in PIPKI-mediated actin reorganization

One main function of PIPKI is actin cytoskeleton reorganization [21, 23, 60, 61]. PIPKI produces PIP_2, which is a key regulator for the recruitment and activity of

many actin- remodeling proteins[54]. Actin comet and foci formation is a readout of actin reorganization in PIPKI-overexpressed cells[64]. I show in this text that the KRHH residues, not charge, are necessary for actin comet/foci formation. This effect is dependent on these residues and not by its mutant protein's inability to localize to the membrane. When a plasma membrane localization signal was added to the KRHH (**A**) mutant, actin reorganization was not rescued. It appears that these four basic residues help regulate PIPKI function. I further investigated their mechanistic role in regulating PIPKI function.

The KRHH residues directly bind PA and are involved in PA stimulation of PIPKI

The generation of PIP_2-rich microdomains is necessary for PIPKI-mediated actin reorganization [54]. PIPKI produces high levels of PIP_2 when stimulated by PA. Although PIPKI may be recruited to the membrane by actin remodelers, I show here that the KRHH residues are involved in PA stimulation and direct PA-binding. Basal kinase activity in the absence of PA is still produced in a KRHH-mutated protein, however, this protein is unable to produce high levels of PIP2 in the presence of PA-containing liposomes. This could be because the protein is unable to directly bind PA, which may recruit and stabilize PIPKI at the membrane. When PA binding was tested using a liposome binding assay, I found that the KRHH residues were unable to bind PA-containing liposomes. An inability to bind to PA explains why PA is not sufficiently recruited to the membrane, is not stimulated by PA, and cannot regulate actin dynamics.

I hypothesize that PIPKIγ regulates actin reorganization through the well-established N-WASP/Arp2/3 pathway. Both PIPKI and N-WASP have been shown to be involved in vesicle motility and actin comet formation [57, 64]. The PIP_2-binding region of N-WASP has been identified [57], as I have done for PA and PIPKIγ here. However, whereas the PA-binding of PIPKIγ is regulated by four residues, N-WASP PIP_2-binding is dependent on charge and PIP_2 membrane density. Although they use different modes by which to recognize and bind their respective lipid, it would be interesting to investigate whether PIPKI actin polymerization activity is dependent on PA molar density as N-WASP is dependent on PIP_2 molar density.

Binding to PA may also change the conformation of PIPKI thereby allowing it to remain at the membrane longer or span a larger area of the membrane where there are more lipid substrates to turnover. Or perhaps binding to PA may cause a change in the protein's orientation. These possibilities would explain the higher turnover rate of PIPKI in the presence of PA. If the PIPKI structure were ever solved, it would be interesting to see the differences in conformation of the lipid-bound and free protein.

A circular regulatory mechanism exists between PLD, PA, PIPKI, and PIP_2 (reviewed in [77]). PLD generates PA, which stimulates PIPKI to produce PIP_2. PIP_2, in turn, is a cofactor for PLD. This positive feedback loop allows for the increased, localized accumulation of both PA and PIP_2 that is important in many cellular functions, including vesicle trafficking and Golgi maintenance [78, 79]. The

PA binding mutant described here can be used to study the intricacies of this pathway and its role in these and other critical cellular functions.

The **A** mutant could be used as a tool to investigate the role of the PA-PIPKIγ relationship in other signaling events, such as endocytosis, membrane ruffling, and secretion. The four KRHH residues identified here may also lead to further analysis of other PA-interacting proteins in an attempt to generate new information and, perhaps, identify a more universal sequence for PA-binding. Identifying the binding constants for the wild type protein as well as the **A** mutant and the double mutants may shed more light on the regulation of PIPKIγ by membrane PA. Does the molar density of PA help regulate PIPKI binding or do membrane size and/or curvature play a more crucial role? The results presented here also rule out the notion that charge alone is responsible for PA-interaction and membrane recruitment.

Su, et al have characterized a PLD-specific inhibitor, FIPI, which can be used to decrease the production of PA at the plasma membrane [80, 81]. Coupled with well-established DAG kinase inhibitors, it will be interesting to see whether the origin of PA has an effect on PIPKIγ activity, localization, and function. Can the KRHH residues distinguish between PLD-produced PA and DGK-produced PA as it does mitoPLD-produced PA? As more details of the regulation of PIPKI become available, we may better begin to understand its roles in development, anaphylaxis, synaptic transmission, and platelet adhesion, all of which have been affected in PIPKI knockout mice.

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