Phosphatidic acid regulation of PIPKI is critical for actin cytoskeletal reorganization®

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Abstract  Type I phosphatidylinositol-4-phosphate 5-kinase (PIPKI) is the main enzyme generating the lipid second messenger phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2], which has critical functions in many cellular processes, such as cytoskeletal reorganization, membrane trafficking, and signal transduction. All three members of the PIPKI family are activated by phosphatidic acid (PA). However, how PA regulates the activity and functions of PIPKI have not been fully elucidated. In this study, we identify a PA-binding site on PIPKIγ. Mutation of this site inhibited the PA-stimulated activity and membrane localization of PIPKIγ as well as the formation of actin comets and foci induced by PIPKIγ. We also demonstrate that phospholipase D (PLD) generates a pool of PA involved in PIPKIγ regulation by showing that PLD inhibitors blocked the membrane localization of PIPKIγ and its ability to induce actin cytoskeletal reorganization. Targeting the PIPKIγ PA-binding-deficient mutant to membranes by a membrane localization sequence failed to restore the actin reorganization activity of PIPKIγ, suggesting that PA binding is not only involved in recruiting PIPKIγ to membranes but also may induce a conformational change. Taken together, these results reveal a new molecular mechanism through which PA regulates PIPKI and provides direct evidence that PA is important for the localization and functions of PIPKI in intact cells.—Roach, A. N., Z. Wang, P. Wu, F. Zhang, R. B. Chan, Y. Yonekubo, G. Di Paolo, A. A. Gorfe, and G. Du. Phosphatidic acid regulation of PIPKI is critical for actin cytoskeletal reorganization. J. Lipid Res. 2012. 53: 2598–2609.

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Inositol phospholipids play an important role in cellular functions, either as precursors of second messengers or by directly interacting with proteins to manage the spatiotemporal organization of key intracellular signal transduction pathways (1–4). The best-studied inositol phospholipid is phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2], which is a critical second messenger regulating a myriad of diverse cellular activities, including actin cytoskeletal reorganization, vesicle trafficking, adhesion, migration, cell proliferation, cell death, and cell metabolism (2, 5, 6). To effectively regulate these disparate cellular events, synthesis of PI(4,5)P2 by phosphatidylinositol phosphate (PIP) kinases must be both spatially and temporally regulated. Two subfamilies of PIP kinases, types I and II, mediate the generation of PI(4,5)P2 from independent pools of substrates, phosphatidylinositol-4-bisphosphate (PI4P) and phosphatidylinositol-5-phosphate (PI5P), respectively (5, 6). The type I PIP kinases (PIPKI, PIPKIβ, and PIPKIγ), as well as their splice variants, have been characterized. Different PIPKI members show some overlapping as well as distinct cellular and biological functions (5–7).

One common function regulated by PI(4,5)P2 and PIPKI isoforms is induction of actin cytoskeletal reorganization, which is critical for cell motility, invasion, pathogen infection, etc. (1, 6–8–10). The increased expression and activity of all three PIPKI members induces a drastic reorganization of actin cytoskeleton: formation of actin comets and foci, structures that are found in cells infected

Abbreviations:  PA, phosphatidic acid; PGDF, platelet-derived growth factor; PI(4,5)P2, phosphatidylinositol-4,5-bisphosphate; PIPKI, type I phosphatidylinositol-4-phosphate 5-kinase; PLD, phospholipase D; PS, phosphatidylserine; WT, wild-type.

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with intracellular pathogens, such as *Listeria, Vaccinia,* and *Shigella* (11, 12), and in cells stimulated with platelet-derived growth factor (PDGF) and pervanadate (13). It has been demonstrated that intracellular pathogens use actin comets and vesicles associated with them for intracellular propulsion (12, 14–16).

In contrast to the widely proposed function of PI(4,5)P2, the molecular pathways regulating PIP kinases as well as the precise mechanisms controlling their catalytic activity still remain largely unknown. A common enzymatic property of all three PIPKI members is that they are strongly stimulated by phosphatidic acid (PA) (17, 18); this stimulation is specific to PA, as other acidic phospholipids, such as phosphatidylserine (PS), has no effect on the activity of PIPKIs (17, 18).

The best-known signaling PA is produced by the hydrolysis of phosphatidylcholine by phospholipase D (PLD) (19–21). Several cellular functions, such as actin cytoskeletal reorganization and vesicle trafficking, are regulated by both PLD and PIPKI (22, 23). Interestingly, PI(4,5)P2 is also an essential factor for PLD activity (22, 23). It has been long proposed that this positive feedback regulation is important for cellular functions. However, this hypothesis is mainly supported by in vitro observations. It is unclear whether PA regulation of PIPKIs occurs in intact cells and whether it is physiologically relevant.

In the current study, we demonstrate that PA regulates the membrane targeting and activity of PIPKI through direct binding to specific basic amino acid residues on its membrane interacting surface. Using the PA-binding-deficient mutant created in this study and pharmacological inhibitors, we further show that PLD-generated PA is required for the formation of actin comets and foci induced by PIPKγ.

**EXPERIMENTAL PROCEDURES**

**General reagents and antibodies**

Cell culture media and sera were from Invitrogen (Carlsbad, CA). DAG kinase inhibitors were from Calbiochem (La Jolla, CA). All other reagents were of analytical grade unless otherwise specified and were from Sigma (St. Louis, MO). All restriction enzymes were from New England Biolabs (Ipswich, MA). T7 antibody was from EMD Chemical (Cincinnati, OH). GFP antibody was from New England Biolabs (Ipswich, MA). T7 antibody was from Abcam (Cambridge, MA). Rhodamine-phalloidin, goat anti-mouse IgG conjugated with Alexa 680, and goat anti-rabbit IgG conjugated with Alexa 680 were from Invitrogen. The PLD inhibitor FIP1 (PLDi-FIPI) originally developed by Novartis (East Hanover, NJ) (24) was provided by Drs. Michael Gelb (University of Washington) and Michael Frohman (Stony Brook University) (25). The PLD inhibitor VU0155056 was from Avanti Polar Lipids (Alabaster, AL).

**Sequence analysis and homology modeling**

Primary sequence alignment of PIPKIβ and the α, β, and γ of PIPKI family members was performed using MultAlin (26). Secondary structure predictions were performed using the programs nnPredict (Expasy Tool) and Phyre (27). The three-dimensional model for a PIPKγ monomer is modeled using homology modeling program MODELER (28) based on the structure of PIPKIβ (Protein Data Bank ID 1B01) (29). The resulting structure is then manually docked on to a hypothetical membrane derived from a previously published POPC/POPG bilayer model (30).

**Construction of plasmids**

Construction of GFP-PIPKγ wild-type (WT) has been previously described (31). All point mutants were generated by site-directed mutagenesis using the QuikChange kit from Agilent Technologies (Santa Clara, CA). The Mem-tagged GFP-PIPKγ and -tKHH/A mutant were generated by adding the membrane-targeting signal from the tyrosine kinase Lyn (MGC/SSRKK) (32) to the N-terminus of PIPKIγ protein. For protein purification, PIPKIγ WT and mutants were cloned into the EcoRI and HindIII sites of the bacterial expression vector pET24 and contained a N-terminal T7 tag and a C-terminal 6xHis tag. All constructs were confirmed by sequencing.

To generate tetracycline-inducible stable cell lines, the coding sequences for GFP-PIPKγ proteins were subcloned from the original pEGFP-C vector into the pxDNA5/FRT/TO vector from Invitrogen. All PIPKIγ WT and mutants are expressed at similar levels compared with WT (supplementary Figs. I–III).

**Bacterial expression and purification of recombinant PIPKIγ proteins**

Recombinant WT and mutant PIPKIγ proteins were expressed in *E. coli* Rosetta 2 cells and purified by Ni²⁺-chelate chromatography according to Qiagen’s manual (Valencia, CA). Proteins were first dialyzed against 20 mM Tris (pH 7.6), 200 mM NaCl, 5 mM β-mercaptoethanol, and then 20 mM Tris (pH 7.6), 100 mM NaCl, and 5 mM β-mercaptoethanol to exchange the buffer and remove the imidazole used in purification. The concentrations of the proteins were measured by Coomassie Plus-200 protein assay reagent from Thermo Scientific (Rockford, IL). All purified recombinant proteins used in our experiments were more than 95% pure, as judged by Coomassie Blue-stained SDS-PAGE. The purified proteins were used within one week.

**Liposome pulldown assay**

The preparation of liposomes has been previously described (33–35). Phosphatidylcholine (POPC), phosphatidic acid (POPA), and Brain PI4P and PI4P were purchased from Avanti Polar Lipids. Large unilamellar vesicles (LUV) were generated by mixing POPC with PI4P (80:20) or POPC and PI4P 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, and then resuspended in 176 mM Sucrose, 20 mM Tris (pH 7.6) at 2 mM total phospholipid concentration.

Hydrated lipids were subjected to at least six cycles of freeze thawing in liquid nitrogen and 37°C water bath before ten cycles of extrusion through a 100 nm membrane filter with a lipid extruder from Northern Lipids (Burnaby, BC, Canada). These 100 nm sucrose-loaded liposomes were collected after ultracentrifugation at 100,000 g, and then resuspended in 20 mM Tris (pH 7.6) and 100 mM NaCl.

Proteins were precleared at 100,000 g to remove any protein aggregates before adding it to the assay. Binding assays were performed by mixing 100 ng of the precleared protein and 400 µM sucrose-loaded liposomes in 20 mM Tris (pH 7.6), 100 mM NaCl, 1 mM DTT, and 1 mM EDTA. After 30 min incubation at room temperature, protein bound to liposomes was recovered by ultracentrifugation at 100,000 g for 30 min. The pellets were then resuspended in SDS-PAGE buffer and resolved by SDS-PAGE. Western blot analysis was performed using primary mouse anti-T7 antibody and Alexa 680 goat anti-mouse secondary antibody. Band intensity was quantified using the LI-COR Odyssey Infrared Imaging System from LI-COR Biotechnology (Lincoln, NE).

**PIPKI kinase activity assay**

Activity assays were performed as described (36) for 15 min at room temperature in 100 µl reactions containing purified.
protein, 400 μM liposome, 20 mM Tris (pH 7.6), 100 mM NaCl, 50 μM ATP, 10 mM MgCl₂, and 5 μCi [³²P]γATP/reaction. Reactions were terminated using 1 M HCl, and the lipid products were extracted with 1:1 chloroform:methanol, washed with 1:1 1 M HC1:methanol, and separated on a 1% potassium oxalate-pregnated Silica Gel H TLC plate (Analtech, Newark, DE) developed in chloroform:methanol:ammonium hydroxide:water (90:10:1:35). The air-dried TLC plate was exposed to autoradiography film overnight. The corresponding product bands from the film were scraped from the TLC plate into scintillation vials, dissolved in Ecolume counting liquid, and quantified on a Beckman Coulter LS 6500 Liquid Scintillation Counter (Indianapolis IN). PI(4,5)P₂ produced 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 counting efficiency). This value was then divided by the amount of protein (ng) used in the assay.

Cell culture and transfection

Cos7 cells were kept in Dulbecco’s modified eagle medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. For transfection, cells grown in 6-well plates or 35 mm dishes (3-4 x 10⁵ cells/dish) were transfected with 1 μg of DNA/dish using the deacetylated poly-ethylenimine reagent (PEI) (37). For inhibitor treatment, Cos7 cells were preincubated with the desired inhibitors or DMSO for 1 h before fixation. To generate tetracycline-inducible stable cell lines, Flp-In T-Rex 293 cells cultured in DMEM containing 10% tetracycline-free fetal bovine serum were transfected with PIPKIγ WT and mutants cloned into pcDNA5/FRT/TO with pOG44 at a ratio of 9:1, and then selected with 100 μg/ml hygromycin for targeted insertion for three weeks. The expression of PIPKIγ proteins in stable cell lines was induced with 1 μg/ml doxycycline for one day. Western blotting analysis confirmed that different PIPKIγ proteins expressed at similar levels (supplementary Fig. III).

Confocal microscopy

Cos7 cells were cultured on cover slips and transfected. Twenty hours after transfection, cells were fixed with 2% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100, and stained with rhodamine-conjugated phalloidin (Invitrogen). Fluorescent images were captured using a Nikon A1 confocal microscope (Melville, NY) and processed using Adobe Photoshop CS 5 (San Jose, CA). All experiments were repeated at least three times. The localization of different PIPKIγ proteins was analyzed in more than 10 cells using NIH Image J program. For each cell, the ratio of plasma membrane and cytosolic PIPKIγ localization was the average of the ratios of pixel intensity on the plasma membrane and that in cytosol (10 pixels from the plasma membrane) from six random areas. For the analysis of actin cytoskeletal reorganization, the number of actin foci and comets was counted in 20 cells for each experiment and presented as the number of foci and comets per cell.

Phosphoinositide analysis

Pi4P and PI(4,5)P₂ were measured in the parental Flp-In T-Rex 293 cells or Flp-In T-Rex 293 cells expressing PIPKIγ WT and mutants using anion-exchange HPLC with suppressed conductivity detection as described before (38, 39).

Statistics

Statistical significance was evaluated by two-tailed Student t-test. All data are shown as mean ± SD.

RESULTS

Identification of potential PA-interacting residues in PIPKIγ

PA-binding proteins typically interact with the negatively charged head group of PA through their positively charged amino acid residues, including lysine, arginine, and histidine (40, 41). This interaction is either mediated by structurally well-defined protein modules in some proteins, such as the PH domain of Sos (35) and the PX domain of p17⁷ PIPKII (42), or by largely unstructured motifs harboring several basic amino acids in such proteins as Raf-1, mTOR, and DOCK2 (41, 43, 44). The crystal structure of PIPKIβ revealed a largely flat surface that binds to the membrane via electrostatic interactions (29). A similar flat surface on PIPKIγs, for which structures do not yet exist, may be important for PA binding since the small headgroup of PA, i.e., a phosphate, may fit well in a shallow pocket on a flat surface. Because PA stimulates the activity of all PIPKI family members but not that of PIPKI or PIPKII members (17, 18), we hypothesized that the potential PA-interacting residues should fit the following three criteria: i) located close to or within the putative flat membrane interacting surface, ii) conserved in the three PIPKI members but not in PIPKIγ, and iii) positively charged.

We chose to use PIPKIγ_1 [Human Genome Variation Society nomenclature; also known as PIPKIγ-87 in previous literature (45)] as the model to study the mechanism by which PA regulates PIPKIγ. PA stimulation of PIPKIγ is the strongest among the three PIPKI family members (18), and PIPKIγ_1 does not contain the C-terminal isoform-specific focal adhesion targeting tail found only in PIPKIγ-90 (31, 46). Primary sequence alignment of PIPKIβ and all three members of PIPKI family (α, β, and γ) revealed that 11 basic amino acid residues on PIPKIγ_1 (K97, R100, H126, H127, H192, K193, K200, R214, K219, K231, and R234), which align to the residues on the flat membrane interacting surface of PIPKIβ, are unique to PIPKIγ (Fig. 1A). We hypothesized that some of these residues are responsible for the PA binding of PIPKIγ. To better confirm our prediction based on the primary sequence alignment, we also performed structure modeling of PIPKIγ. In the absence of known PIPKI structure, we performed homology modeling based on the published PIPKIβ structure (Protein Data Bank ID 1BO1) (29), which has high homology with PIPKIγ (Fig. 1A). The structure modeling supported that PIPKIγ also has a flat membrane interacting surface that is rich in basic amino acid residues, including all but two of those identified by primary sequence alignment (Fig. 1B).

Importantly, the two neighboring histidine residues on a flexible loop, H126 and H127, lie very close to our hypothetical model membrane. Although this loop points away from the bilayer in our rigid-body model, its predicted flexibility may allow it to adopt a different conformation upon protein motion and/or binding to a negatively charged bilayer. We reasoned that this loop might be useful for a regulated and specific binding of small membrane-bound molecules, such as PA.
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H127 (KRHH); mutant B contained H192, K193, and K200 (HKK); and mutant C contained R214, K219, K231, and R234 (RKKR). Although residues R214 and K219 were not contained within the potential membrane interacting surface in the modeled structure (Fig. 1B), they were included in mutant C in case our structure modeling

The potential involvement of the above-mentioned basic residues in PA binding was tested by substituting them with uncharged alanine residues in PIPKIγ by sequence analysis. (A) Primary sequence alignment of PIPKI members (α, β, γ) and PIPKIβ. Conserved amino acids are highlighted in blue (75% conserved) and red (100% conserved). Green arrows and bars indicate secondary structure elements (β strands and α helices, respectively) and corresponding amino acids for PIPKI (predicted using Expasy and Phyre prediction software) that also correspond to the same PIPKIβ secondary structure elements. (B) A homology model of PIPKIγ based on the structure of PIPKIβ (PDB code 1BO1). The secondary structure of the protein is yellow, and the catalytic residues are red. Groups of residues subjected to alanine substitution in the current study are shown in stick model and colored in dark green and blue (group A, residues K97, R100, H126, and H127); light green (group B, residues H192, K193, and K200); and cyan (group C, residues R214, R219, K231, and R234). A monolayer of hypothetical bilayer is shown as a surface colored from white (hydrophobic core) to blue (polar head group region).
prediction did not reflect the true structure of the protein. Western blotting analysis of these mutants showed that they express at the level similar to WT protein (supplementary Fig. 1), suggesting that introducing these point mutations were not likely to cause a structural change. The ability of both WT and mutant PIPKIγ-i1 (hereinafter PIPKIγ) to bind PA was then determined by a sedimentation assay using recombinant proteins purified from E. coli and synthetic liposomes. As expected, we found that PIPKIγ WT binds to PA-containing liposomes. As predicted by our model, PA binding was dramatically reduced by the mutations in mutant A (KRHH/A) (only around 14% of liposome binding was left) but was not affected by those in mutant B or C, suggesting that the residues in mutant A are important for PA binding (Fig. 2A). To further define the key residues in mutant A (KRHH/A), proteins with two amino acid mutations, K97A/R100A (KR/A) and H126A/H127A (HH/A), were tested for their PA binding. Both mutants showed a reduction in PA binding (74% and 38% liposome binding, respectively); however, the reduction in binding was less than with mutant A (Fig. 2B). These results suggest that efficient PA binding requires the coordination of all four residues in group A (KRHH). This result is in line with previous the conclusion that many PA-binding proteins bind to PA through similar multivalent interactions (41, 43, 44).

To further confirm the specificity of the PA binding through the KRHH residues, we examined whether PIPKIγ binds to PA in a concentration-dependent manner and whether the KRHH residues are mainly responsible for PA binding rather than other acidic phospholipids, such as PS, and the catalytic substrate of PIPKIγ, PI4P. The purified PIPKIγ WT and mutant A were tested for their interaction with different concentrations of sucrose-loaded liposomes containing PA, PS, or PI4P. Similar to the findings above, the binding of mutant A to PA liposomes was significantly decreased compared with WT (Fig. 2C). On the other hand, both WT and the mutant A bound to PS and PI4P liposomes at similar levels (Fig. 2C). These data support our hypothesis that PA binding of PIPKIγ is mediated by specific amino acids, which are independent of or less critical for residues mediating the interactions with other acidic phospholipids and substrate binding.

**PA binding is required for PA-stimulated but not basal PIPKI activity**

PA regulation is a unique property of PIPKI (17, 18). Furthermore, the substrate specificity of PIPKI and PIPKII can be switched by swapping one amino acid in the activation loop (E411 in PIPKIγ) (17, 48). The activation loop is located approximately 280 residues downstream of the PA-binding site in the primary sequences (Fig. 1A) and at the opposite side of the PA-binding site on the modeled three-dimensional structure of PIPKIγ (more than 30 Å away) (Fig. 1B). These findings suggest a regulatory role for PA binding of PIPKI rather than direct roles in catalytic specificity and basal activity of PIPKIs. To investigate the role of PA binding in the regulation of PIPKI activity, PI(4,5)P2 production was measured using liposomes in the presence or absence of PA. The PA-binding-deficient mutant A still maintained a comparable activity to the WT protein (Fig. 3). In contrast, it lost the PA-stimulated activity (Fig. 3), suggesting that direct PA binding is critical for the PA-stimulated activity of PIPKIγ.

**Membrane localization of PIPKIγ and its actin cytoskeletal reorganization activity are dependent on PA binding**

The PIPKI members are reported to localize to the plasma membrane and/or intracellular vesicles (2, 5, 6). When overexpressed, these enzymes induce drastic reorganization of the actin cytoskeleton (13, 18, 49). To assess the contribution of PA binding to PIPKI membrane localization and actin cytoskeletal reorganization activity, we utilized a GFP-tagged PIPKIγ construct (31) and subcloned all mutants described above into this plasmid. Western blotting analysis confirmed that WT and all mutants used in the current study were expressed at similar levels (supplementary Fig. II), suggesting that these point mutations do not induce deleterious effects on protein structure. Similar to previous reports, transiently transfected GFP-PIPKIγ localized to the plasma membranes as well as intracellular vesicles in Cos7 cells and induced the formation of two kinds of filamentous actin (F-actin) structures resembling those found in pathogen-infected cells (12–15): comets (phalloidin-stained F-actin structure with a head and a tail) and foci (F-actin-labeled round spots) (Fig. 4). The formation of actin comets and foci has been known to be dependent on PI(4,5)P2 and PI(4,5)P2-regulated actin regulatory proteins (50, 51). Although localized to the same membrane structures as WT protein, the kinase-inactive PIPKIγ mutant D253A (46) failed to induce the formation of actin comets and foci, and the number of comets and foci were similar to those in non-transfected cells (Fig. 4), confirming their formation is dependent on PI(4,5)P2 production (Fig. 4A–C). The PA-binding mutant A (KRHH/A) was diffused in the cytosol and failed to induce the formation of either comets or foci (Fig. 4A–C). Corresponding to their PA-binding capacity, the HH/A and KR/A double mutants also showed decreased membrane localization and comet and foci formation (Fig. 4). In contrast, mutants B and C, which bind PA normally, retained membrane localization and similar quantities of comets and foci compared with the WT protein (Fig. 4A–C). Taken together, our results suggest that PA binding is critical for the membrane localization of PIPKIγ and its ability to induce the formation of actin comets and foci.

We next measured PI4P and PI(4,5)P2 levels using HPLC with suppressed conductivity detection (38, 39). To better compare the contribution of exogenous PIPKIγ proteins on the background of other PI(4,5)P2-generating enzymes in cells and to minimize the toxicity of long-term PIPKIγ overexpression, we established several stable cell lines expressing PIPKIγ WT and mutants under the control of a tetracycline-inducible promoter using the Flp-In T-Rex system from Invitrogen. Western blotting analysis confirmed similar expression levels of PIPKIγ proteins in
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Both PA-dependent and PA-independent electrostatic interactions may regulate the functions of PIPKI in intact cells.

A recent study has shown that nonspecific electrostatic interaction between acidic phospholipids and PIPKI is important for membrane association of the protein (52). Based on our findings above, charge alone does not seem to play a significant role in PIPKI regulation since the reduction of charges did not correspond to the reduction of PA binding, kinase activity, membrane targeting, or formation of actin comets or foci. The mutants in groups A (KRHH/A), HH/A, KR/A, B (HKK/A), and C (RKKR/A), had a charge reduction of 4, 2, 2, 3, and 4, respectively. However, PA regulation of PIPKI was mainly mediated by the residues K97, R100, H126, and H127 (Figs. 2–4).

The similarly decreased charge in mutants B and C did not different cell lines with doxycycline induction (supplementary Fig. III). As expected, overexpression of PIPKI WT led to a significant increase of PI(4,5)P2 level compared with that in the parental cells (about 220% change) and to a reduction of its enzymatic substrate PI4P level (about 50% reduction) (Fig. 4D). In contrast, PI(4,5)P2 and PI4P levels in the mutant A-expressing cells were only raised slightly (about 110%) (Fig. 4D). Consistent with their abilities of binding to PA and inducing the formation of actin comets and foci, PI(4,5)P2 and PI4P levels in the mutant B- and C-expressing cells were comparable to those in WT-expressing cells (Fig. 4D). This result provides evidence that PA regulation is also important for PIPKI activity in intact cells and that the change in PI(4,5)P2 levels is responsible for actin reorganization in cells that express different PIPKI isoforms.

Fig. 2. Identification of a PA-binding site on PIPKI by liposome pulldown assays. (A) Mutations of residues in group A disrupted the PA binding of PIPKI. The PIPKI residues in groups A (KRHH), B (HKK), and C (RKKR), which are marked in Fig. 1, were mutated to alanine. The purified recombinant WT or mutant protein was incubated with sucrose-loaded liposomes containing 10% PA. After centrifugation, bound proteins in the pellets were detected by Western blot using a T7-tag antibody. The inputs are 10% of proteins used for binding. Quantification of relative binding of the mutants to PA-liposomes compared with WT is shown below the blot. N = 3; **P < 0.0005 versus WT. (B) Binding between PA and PIPKI is through multivalent interaction. Mutations of HH and KR residues in group A to alanine also reduced PA binding of PIPKI. Quantification of relative binding of the mutants to PA-liposomes compared with WT is shown below the blot. N = 3; *P < 0.05 versus WT; **P < 0.004 versus WT. (C) Specific PA binding of PIPKI. The purified recombinant WT or mutant A was incubated with different concentrations of sucrose-loaded liposomes containing either 10% PA (left), 10% PS (middle), or 10% PI4P (right). After centrifugation, bound proteins in the pellets were detected by Western blot. The Western blot is a representative of three experiments. The dose curves are quantification of relative binding of the mutants to liposomes compared with WT from three independent experiments.
affect the membrane localization and actin cytoskeletal reorganization activity, supporting the important role of specific PA binding in PIPKι regulation. However, these observations do not rule out the possibility that both specific and nonspecific electrostatic interactions contribute to the regulation of PIPKι. Membrane targeting of many proteins often requires coincidence detection of different lipids and proteins by individual domains and specific and nonspecific electrostatic interactions (40). To determine whether overall protein charge was also responsible for the regulation of PIPKι in our experimental system, three additional mutants were generated, containing different combinations of mutants A, B, and C, to further reduce the positive charges on the predicted membrane interacting surface of PIPKι. The new “charge mutants,” A+B, A+C, and B+C, resulted in decreases of positive charge of 7, 8, and 7, respectively. The in vitro liposome pulldown experiment revealed that both charge mutants containing A (A+B and A+C) had similar PA binding compared with mutant A, suggesting that removal of additional positive charges from these two mutants did not further reduce the PA binding of mutant A (KRHH) (Fig. 5). Furthermore, a decrease of 7 positive charges at sites away from group A residues did not affect the PA binding of mutant B+C (Fig. 5). These results support our finding that PIPKι specifically binds to PA through the KRHH residues in group A.

When expressed in Cos7 cells, the GFP-tagged mutants A+B and A+C localized to the cytosol and failed to induce the formation of actin comets and foci, similar to mutant A (Fig. 6). The GFP-B+C mutant behaved slightly differently from mutant B and mutant C, separately. While a portion of proteins still localized to membranes, there was a decrease of plasma membrane localization (Fig. 6). Consistent with membrane localization, the number of actin comets was also slightly decreased in GFP-B+C-expressing cells (Fig. 6). Because the level of PA binding of mutant B+C was comparable to the WT protein in the in vitro reconstituted liposome binding assay, the decreases in the membrane localization and actin reorganization activity of mutant B+C in intact cells were most likely caused by the reduction of general electrostatic interaction between membranes and PIPKι. Taken together with our finding that the PA-binding-deficient mutant A binds to PS and PI4P normally (Fig. 2C), our results suggest that both PA-dependent and PA-independent electrostatic interactions regulate PIPKι in intact cells.

**PIPKι activity is regulated by PA generated from PLD**

The best-known source of signaling PA is generated by PLD, which is involved in the regulation of actin cytoskeletal reorganization (21). To investigate whether PLD activity regulates PIPKι functions, we treated cells expressing GFP-PIPKι with PLD inhibitors. Consistent with the findings using the PA-binding-deficient PIPKι mutants, PLD inhibitors developed by two different sources, PDLi-FIPI (24, 25) and PDLi-Avant (purchased from Avanti Polar Lipids) (53), prevented the membrane association of PIPKι and significantly blocked the formation of actin comets and foci induced by PIPKι (Fig. 7). Thus our data provide the first evidence that PLD-generated PA is required for membrane localization and functions of PIPKι in intact cells.

**PA binding is required for both membrane localization and actin reorganization activity of PIPKι**

Phospholipid binding is important for both membrane recruitment and activation of many proteins (40). Alternatively, phospholipid binding may also regulate the activity of proteins through a membrane targeting-independent mechanism (40, 54). To determine whether PA-mediated membrane targeting alone is sufficient for PIPKι activation, PIPKι proteins were targeted to membranes through a PA-independent mechanism by adding the membrane targeting motif of the tyrosine-protein kinase Lyn to their N-termini (Mem-PIPKι) (32). Both Mem-PIPKι and Mem-KRHH/A displayed subcellular localizations to plasma membranes and vesicles similar to the WT protein. The addition of the membrane targeting motif did not affect the actin cytoskeletal reorganization activity of WT protein or the PI(4,5)P2 and PI4P levels (Fig. 8A–C). In contrast, although Mem-KRHH/A was successfully localized to membranes, it failed to induce the formation of actin comets and foci (Fig. 8A–C). Measurement of PI(4,5)P2 and PI4P levels confirmed that targeting KRHH/A to membranes was unable to increase its enzymatic activity (Fig. 8D). This result suggests that PA binding is critical for both membrane targeting and activation of PIPKι. The exact mechanism through which PA regulates PIPKι still remains to be determined. It is likely that PA binding induces a conformational change in PIPKι or enhances its binding to other regulators in addition to targeting the protein to membranes.

**DISCUSSION**

On the basis of sequence analysis, structure modeling, and liposome pulldown assays, we demonstrate that protein residues H126 and H127, which are likely located on
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a flexible loop, along with the nearby K197 and R100 are key residues for specific PA binding by PIPKIγ. This conclusion is also supported by two other evidences: i) mutant A (KRHH/A) retained its basal catalytic activity even when PA-stimulated activity was lost, suggesting that its substrate binding ability is not affected by these mutations; and ii) charge changes on the other PIPKIγ mutants (groups B and C) did not correspond to their PA binding ability, suggesting that charge alone is not the determining factor for PA binding of PIPKI. Two previous studies also reported different PA-binding sites on PIPKIγ. Using a 96-well plate coated with phospholipids, the purified mouse PIPKβ (human PIPKα) was reported to bind PA through its C terminus (55). However, this result did not explain why

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**Fig. 4.** PA binding is critical for plasma membrane localization and actin cytoskeletal reorganization activity of PIPKIγ. (A) Cos7 cells were transfected with GFP-PIPKIγ WT, the catalytically inactive mutant D253A, and other alanine mutants. Twenty hours after transfection, cells were fixed and stained with rhodamine-conjugated phalloidin. Actin comets and foci are marked with arrowheads and arrows, respectively, in the PIPKIγ-transfected cells. Fluorescent images were captured with a Nikon A1 confocal microscope. The same pictures at larger sizes are included in supplementary Figs. IV and V. Untransfected cells are marked with an asterisk. (B) Quantitation of the plasma membrane/cytosol PIPKIγ ratio (see Experimental Procedures). N = 11–14; **P < 0.00001 versus WT. (C) Quantification of the number of comets (blue) and foci (red) in cells transfected with GFP-PIPKIγ WT and mutants (see Experimental Procedures). N = 20; **P < 0.05 versus WT; ***P < 0.001 versus WT; **P < 0.02 versus WT. (D) Phospholipid levels in stable cells expressing PIPKIγ WT and mutants. The expression of PIPKIγ proteins was induced for 1 day using 1 g/ml doxycycline (supplementary Fig. III). The change of phospholipid levels was compared with those in the parental cells. N = 3; **P < 0.03 versus PI4P in parental cells; **P < 0.003 versus PI5P in parental cells.
For example, both histone fold and PH domains on Sos bind to PA (54).

It has become increasingly apparent that most proteins are directed to their functional destination by more than one targeting determinant (40). The coexistence of multiple signals ensures the specificity of protein targeting within the cell. The membrane localization and activity of PIPKI may depend on several factors, including substrate, regulatory proteins, and phospholipids. A single mutation (E362A) in the activation loop of the PIPKI altered its substrate selectivity and caused it to be displaced from the plasma membrane (47, 48). A point mutation on the Rac interaction site also disrupted the membrane localization of PIPKI (57). Less is known about how the localization and function of PIPKI are regulated by phospholipids. It has been reported that a nonspecific electrostatic interaction with the inner surface of the plasma membrane contributes to the membrane binding of PIPKI (52). Our data now show that PA binding plays a critical role in the membrane localization and activity of PIPKI. How PA and other PIPKI regulators are coordinated to control the localization of enzymes at the membranes and regulate PI(4,5)P2 synthesis is unclear. Different combinations of regulators may target PIPKI members to various subcellular localizations, where they perform member-specific cellular functions. The PA-binding-deficient mutant identified in the current study, mutant A (KRHH/A), will be a useful tool to dissect the diverse regulation and functions of PIPKI proteins.

A rather surprising finding was that targeting the PA-binding-deficient mutant KRHH/A to the membrane did not change the PA stimulation of the enzymes in an earlier report (18). In another study, by assessing the membrane translocation of human PIPKIβ in Cos7 cells, a PA-binding site was mapped to amino acid residues 209–215 (56), which are very close in proximity to the substrate (PI-4P) binding site. The different PA-binding sites identified by us and other groups might have resulted from the use of different methods to study PA-PIPKI interaction. Although it is believe that the liposome system used in our study is the best way to mimic cell membranes in vitro, it is also possible that there are multiple PA-binding sites on PIPKI.

Deleting the C terminus of the three PIPKI members did not change the PA stimulation of the enzymes in an earlier report (18). In another study, by assessing the membrane translocation of human PIPKIβ in Cos7 cells, a PA-binding site was mapped to amino acid residues 209–215 (56), which are very close in proximity to the substrate (PI-4P) binding site. The different PA-binding sites identified by us and other groups might have resulted from the use of different methods to study PA-PIPKI interaction. Although it is believe that the liposome system used in our study is the best way to mimic cell membranes in vitro, it is also possible that there are multiple PA-binding sites on PIPKI.

Fig. 5. Surface charge does not contribute to the PA binding of PIPKI. (A) Purified mutants containing different combinations of mutants A, B, and C were tested for their PA-binding ability using liposome pulldown assay as in Fig. 2. The bound proteins in pellets were detected by Western blot using a T7-tag antibody. Inputs are 10% of proteins used for binding. (B) Quantification of relative binding of the mutants to PA-liposomes compared with WT. N = 3; **P < 0.005 versus parental cells.

Fig. 6. Both PA-dependent and PA-independent electrostatic interactions contribute to the functions of PIPKI. (A) Membrane localization of the indicated GFP-PIPKI mutants and their ability to induce the formation of actin comets and foci. The same picture at a larger size is included in supplementary Fig. VI. (B) Quantitation of the plasma membrane/cytosol PIPKI ratio. N = 11–13; **P < 0.0001 versus WT. (C) Quantification of comets and foci in cells transfected with GFP-PIPKI WT and mutants. N = 20; *P < 0.05 versus WT; **P < 0.01 versus WT; ***P < 0.02 versus WT.
mediated by PIPKI, induction of actin comets and foci. This differs from many other proteins whose ability to interact with membranes is a key regulatory step in controlling their activity. It is likely that PA binding does not only target PIPKI to membranes but also induces a conformational change required for the activation of the enzyme. Interestingly, two key PA-binding residues, H126 and H127, are located in a rather flexible loop on the modeled PIPKI structure, implying a capacity for potential conformational changes upon PA binding. A similar PA regulation of protein activity has been recently reported for Sos (54). While the binding of the PH domain of Sos to PA is critical for the ligand-induced membrane recruitment of Sos and, hence, for Sos-mediated Ras activation (35), the binding of the histone fold domain to PA activates Sos through a conformational switch independent of its membrane binding (54). It is possible that both PA and nonspecific electrostatic interaction are required for the initial membrane recruitment of PIPKI and that only PA binding is required for the stabilization of membrane localization and activation of PIPKI.

The formation of actin comets and foci may represent a mechanism utilized by cells to transport membrane-bound organelles under both physiological and pathological conditions. For example, Way and colleagues observed the propulsion of Golgi-derived endosomes by spontaneously formed “little actin tails” in HeLa cells (11, 12). The formation of PI(4,5)P2-dependent actin comets and foci were also reported in cells stimulated with PDGF and pervanadate (13), in the polarized biosynthetic traffic in epithelial cells (58), and in the internalization of macropinosomes forming from membrane ruffles (59). Many pathogens are known to hijack the host’s cytoskeletal machinery and use actin comets for their intracellular movement. Some examples include Simian Virus 40, Listeria, Vaccinia, Shigella, and enteropathogenic E. coli (11, 12, 14–16). Previous studies have demonstrated that actin polymerization regulated by PI(4,5)P2, Nck, and WASP are key in the formation of actin comets and foci (13, 50, 51, 60). Our current findings have demonstrated that PA generated from PLD regulates the activity of PIPKI in intact cells and that this signaling pathway is critical for the PI(4,5)P2-mediated formation of comets and foci. PI(4,5)P2 is also an essential factor for the activation of PLD (34, 61). The existence of the PLD (PA)-PIPKI [PI(4,5)P2] positive signaling feedback loop may provide cells a quick way to generate sufficient PI(4,5)P2 at the right subcellular locations when rapid actin dynamics are crucial under acute conditions, such as bacterial infection. Furthermore, phospholipid signals generated on moving vesicles might provide a precise control of the site of actin polymerization, which is critical for directional vesicle movement in the actin comet-mediated cellular processes.

In summary, we have identified a PA-specific interacting site on PIPKI. Our data also demonstrate that PLD-generated PA is required not only for the membrane targeting of PIPKI but also for its activation. These results provide direct evidence that the PLD (PA)-PIPKI [PI(4,5)P2] signaling feedback loop plays a central role in cytoskeletal dynamics in intact cells. Future studies of these phospholipid signals could provide great insight and understanding of fundamental cytoskeletal dynamics and vesicle trafficking.

Fig. 7. The PA that regulates PIPKIγ functions is generated by PLD. (A) Cos7 cells transfected with GFP-PIPKIγ were treated with either PLD inhibitor developed by Novartis (PLDi-FIPI) or PLD inhibitor from Avanti Polar Lipids (PLDi-Avanti). Twenty hours after transfection, cells were fixed and stained with rhodamine-conjugated phalloidin. Fluorescent images were captured with a Nikon A1 confocal microscope. The same picture at a larger size and pictures of bigger fields, respectively, are included in supplementary Figs. VII and VIII. (B) Quantitation of the plasma membrane/cytosol PIPKIγ ratio. N = 10–11; **P < 0.0001 versus DMSO control. (C) Quantification of comets and foci in cells transfected with GFP-PIPKIγ WT and mutants. N = 20; ##P < 0.0008; ***P < 0.0007 versus DMSO control.

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Fig. 8. Targeting the PA-binding-deficient mutant A (KRHH/A) to membranes is unable to induce the formation of actin comets and foci. (A) Cos7 cells were transfected with either WT or the PA-binding-deficient mutant A (KRHH/A) fused to a membrane-targeting signal derived from Lyn (Mem-WT and Mem-A). Twenty hours after transfection, cells were fixed and stained with rhodamine-conjugated phallolidin. Fluorescent images were captured with a Nikon A1 confocal microscope. The same picture at a larger amine-conjugated phalloidin. Fluorescent images were captured with a Nikon A1 confocal microscope. (B) Quantification of the plasma membrane/cytosol PIPKl ratio. Mem-WT, N = 14; Mem-A, N = 10. (C) Quantification of comets and foci in cells transfected with Mem-PIPKl WT and Mem-KRHH/A. N = 20; **P < 0.004; ***P < 0.004 versus DMSO control. (D) Phospholipid levels in stable cells expressing Mem-PIPKl and Mem-KRHH/A. The expression of PIPKl proteins was induced for 1 day using 1 μg/ml doxycycline (supplementary Fig. III). The change of phospholipid levels was compared with the same parental cells in Fig. 4D. N = 3; **P < 0.002 versus the same parental cells in Fig. 4D.

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