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Identification and Characterization of a Phosphoinositide Phosphate Kinase Homolog*

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Phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) plays a central role in regulating the actin cytoskeleton as a substrate for phosphoinositide 3-kinase and phospholipase C as well as by binding directly to proteins that control the processes of actin monomer sequestration, filament severing, capping, nucleation, cross-linking, and bundling (Ma, L., Cantley, L. C., Janmey, P. A., and Kirschner, M. W. (1998) *J. Cell Biol.* 140, 1125–1136; Hinchliffe, K. (2000) *Curr. Biol.* 10, R104–R1051). Three related phosphatidylinositol 4-phosphate 5-kinases (PI(4)P 5-kinases) have been identified in mammalian cells (types Iα, Iβ, and Iγ) and appear to play distinct roles in actin remodeling. Here we have identified a fourth member of this family by searching the human genome and EST data bases. This new protein, which we have designated phosphatidylinositol phosphate kinase homolog (PIPKH), is expressed at relatively high levels in brain and testis. Immunoprecipitates of PIPKH expressed in mammalian cells contain PI(4)P 5-kinase activity, but this activity is not affected by mutations in residues that inactivate other type I PI(4)P 5-kinases. We show that the PI(4)P 5-kinase activity in PIPKH immunoprecipitates can be explained by the ability of PIPKH to heterodimerize with other type I PI(4)P 5-kinases. Transfection of 293t cells with PIPKH resulted in >8-fold increase in total phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$) without a significant net increase in total $PI(4,5)P_2$. When coexpressed with PIPKH, green fluorescent protein (GFP) fusion construct of the pleckstrin homology domain from Bruton's tyrosine kinase (GFP-BTK-PH) localized in intracellular vesicular structures, suggesting an unusual intracellular site of $PI(3,4,5)P_3$ production. Finally, expression of PIPKH induced the reorganization of actin from predominantly stress fibers to predominantly foci and comets similar to those observed previously in cells infected with the intracellular pathogen *Listeria* or transfected with recombinant PIPKIα. These results suggest that PIPKH acts as a scaffold to localize and regulate type I PI(4)P 5-kinases and the synthesis of $PI(3,4,5)P_3$.

Control of cellular functions involving membrane trafficking and adhesion requires rapid, reversible, and localized assembly of the actin cytoskeleton as well as of other functional complexes such as focal adhesions (1, 3–6). These assemblages of structural and signaling proteins provide a delimited zone of infrastructure, mechanical support, or directionality for dynamic membrane processes. Rapid and reversible assembly of the cytoskeletal framework for membrane vesicle formation, endo- and exocytosis, and cell migration is regulated by the generation of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$)[1] by phosphatidylinositol phosphate kinases (PIPKs) (5). It is now well established that this phosphoinositide is a signaling molecule in its own right, acting through several actin-binding proteins, members of the Wiscott-Aldrich Syndrome protein (WASP) family of proteins, and the Arp2/3 complex (7), as well as by controlling the localization of a subset of proteins containing pleckstrin homology (PH) domains. Dynamin, a large GTPase that is required for fission of nascent vesicles from Golgi and plasma membranes, binds to $PI(4,5)P_2$ by its PH domain and is found at actin-rich sites in peripheral ruffles of eukaryotic cells as well as in actin comets and tails generated by the intracellular pathogen *Listeria* or by overexpression of type I PIPKs (5, 8, 9). In addition, $PI(4,5)P_2$ is converted by phosphoinositide 3-kinases to $PI(3,4,5)P_3$, which can activate members of the Rho/Rac/CDC42 family of GTP-binding proteins to initiate actin cytoskeletal remodeling (1).

The PIPKs compose two families of phosphoinositide kinase, designated types I and II; type I PIPKs are phosphatidylinositol 4-phosphate 5-kinases, and type II PIPKs are phosphatidylinositol 5-phosphate 4-kinases (10). The three isoforms of phosphatidylinositol 4-phosphate 5-kinase, designated types Iα, Iβ, and Iγ, are responsible for the bulk of $PI(4,5)P_2$ synthesis in metazoan cells (2). $PI(4,5)P_2$ synthesis by the type I PIPKs is regulated by members of the Rho/Rac/CDC42 family of GTP-binding proteins (6, 11), as well as by small G protein ADP-ribosylation factors (ARFs) such as ARF-6 (12). The predominant splice variant of type IγPIPK, the major isoform found in

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY376879.

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[1] The abbreviations used are: $PI(4,5)P_2$, phosphatidylinositol(4,5)-bisphosphate; $PI(3,4,5)P_3$, phosphatidylinositol(3,4,5)trisphosphate; PI(5)P, phosphatidylinositol(5)phosphate; PI(4)P, phosphatidylinositol-(4)phosphate; PI(3)P, phosphatidylinositol(3)phosphate; PIPK, phosphatidylinositol phosphate kinase; PIPKH, phosphatidylinositol phosphate kinase homolog; BTK, Bruton's tyrosine kinase; PH, pleckstrin homology; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; ARFs, ADP-ribosylation factors; GFP, green fluorescent protein; EGFP, enhanced GFP; GST, glutathione *S*-transferase, MES, 4-morpholineethanesulfonic acid; WASP, the Wiscott-Aldrich Syndrome protein; NFDM, nonfat dry milk; EST, expressed sequence tag; HA, hemagglutinin; 3-PAP, 3-phosphatase adaptor protein.

brain, is recruited to focal adhesions and activated by an interaction with talin (4, 13). This isoform is expressed specifically in brain and is the major PI(4,5)P_2 synthesizing enzyme at the synapse, where it is concentrated (14). In comparison to that of the type I PIPKs, the regulation and signaling functions of the type II PIPKs are less well understood (10).

We have identified a gene on human chromosome 9 encoding a protein homologous to the known (type I and type II) PIPKs by searching the translated human genome data base for the sequence DYSLL, which is conserved in all type I as well as type II PIPKs. The sequence is located in the N-terminal portion of the catalytic core (15). Within this sequence, Asp^{278} and Leu^{282} of PIPKIIβ are predicted to interact with lipid and ATP substrate. Murine and human cDNAs were subsequently identified in the EST data base, the endogenous protein identified in brain membrane extracts, and the recombinant proteins characterized in mammalian and bacterial expression systems. This protein lacks detectable lipid kinase activity but associates with type I PIPKs *in vivo* and appears to act as a scaffold for localization of PIPK activity.

EXPERIMENTAL PROCEDURES

Materials—Synthetic PI(3)P, PI(4)P, PI(3,4$P)_2$, PI(5)P, and PI(3,5)P_2 were obtained from Echelon Research Laboratories (Salt Lake City, UT). PI, PI(4,5)P_2, and PI(3,4,5)P_3 were obtained from Avanti Polar Lipids. DNA transfection was performed with LipofectAMINE PLUS reagent according to the manufacturer's recommendation.

PIPK Expression—The EcoRI-ApaI fragment of mouse brain cDNA clone A330090A02 (generously provided by the RIKEN Tsukuba Institute (Genome Exploration Group, RIKEN Genomic Science Center, Genome Science Laboratory)), containing the longest open reading frame, was inserted into the EcoRI-ApaI site of pcDNA3 for mammalian expression. The coding region was amplified by PCR employing *Pfu* DNA polymerase and inserted into pGEX4t3 (EcoRI-XhoI site), pFLAGCMV2 (EcoRI-BamHI site), and pBlueBacHis2a (BamHI-SalI site) for GST-tagged, FLAG-tagged, and His-tagged expression, respectively; all epitope tags were N-terminal. Oligonucleotide primer sequences are as follows: 5′-CAGAATTCGGCCACACCAAGCCTTAGG-3′ (sense) and 5′-GTTCCTCGAGTGAGTCATTCTGTGTGCACC-3′ (antisense) for pGEX4t3; 5′-CAGAATTCGGCCACACCAAGCCTTAGG-3′ (sense) and 5′-CGGGATCCAAGGGAAGAAGGCAGACACG-3′ (antisense) for pFLAGCMV2; and 5′-CAGGATCCGCCACACCAAGCCTTAGG-3′ (sense) and 5′-GTTCCTCGAGTGAGTCATTCTGTGTGCACC-3′ (antisense) for pBlueBacHis2a. FLAG-PIPKH and GST-PIPKH were purified by attachment to anti-FLAG (M2)-agarose and glutathione-agarose beads, respectively.

Substitution of amino acid residues known to be necessary for catalytic activity of the known PIPKs were introduced into PIPKH by mutating Lys^{155} to Arg, and by mutating Asp^{281} to Ala using the QuickChange site-directed mutagenesis kit (Stratagene). Oligonucleotide primer sequences are as follows: 5′-GCGCTTCTTTGTGAGGACCCAACGGCGCCACG-3′ (sense) and 5′-CGTGGCGCCGTTGGGTCCTCACAAAGAAGCGC-3′ (antisense) for $155^{Lys\rightarrow Arg}$, and 5′-GGTGAATGTACTGGCTTACAGCCTCCTGGTGGCC-3′ (sense) and 5′-GGCCACCAGGAGGCTGTAAGCCAGTACATTCACC-3′ (antisense) for $281^{Asp\rightarrow Ala}$. pFLAG-CMV2-PIPKH was transcribed and translated *in vitro* using the TNT rabbit reticulocyte coupled transcription/translation system (Promega) and [^{35}S]methionine to test for its ability to bind specifically various phospholipids arrayed on PVDF membranes. *In vitro* translated PIPKH was applied to phospholipids arrayed on PVDF membranes using a slot blot apparatus; in this system, proteins binding to phospholipid spots are detected by ^{35}S autoradiography.

PIPK Assay—PIPK activity was assayed in cell lysates and immunoprecipitates as described previously (30). Phosphatidylserine was employed as carrier lipid in all assays.

Tissue Distribution—Analysis of PIPKH expression was performed by Northern and Western analyses by standard methods. The mouse Multiple Tissue Northern blot was obtained from Clontech and probed with full-length A330090A02. For Western analysis, lysates of frontal lobe from rat, mouse, and human brain were obtained by homogenization in buffer A (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml aprotinin, 10 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 mM sodium orthovanadate, 10 mM NaF, 10 mM β-glycerol phosphate) followed by centrifugation at 40,000 $\times$ *g* to remove membranes and cytoskeleton. The membranes were extracted with 1 M NaCl and then with 1% Triton X-100. The cytosolic, salt-extracted, and detergent-extracted proteins were separated by electrophoresis on a 10% SDS-polyacrylamide gel. Lysates of 293t human embryonic kidney cells transfected with FLAG-PIPKH or with empty vector were run in parallel.

Antiserum Production—Two rabbit polyclonal antisera were raised against a keyhole limpet hemocyanin-linked peptide corresponding to the 15 C-terminal residues (CYARRLSRWAEVHTE) of the mouse sequence. Serine was substituted for the cysteine residue in position 7 of this sequence in order to prevent dimerization. The antisera were produced by Research Genetics (Huntsville, AL) and were designated 61434 and 61435, respectively. The antisera employed were from the third bleed of New Zealand White rabbits and were titered by enzyme-linked immunosorbent assay with free peptide bound in solid phase (1 μg/well). Titers were expressed as the reciprocal of the serum dilution resulting in an A_{405} of 0.2, using biotinylated anti-rabbit IgG, horseradish peroxidase-streptavidin conjugate, and ABTS. The 10-week titers of antisera 61434 and 61435 were 55795 and 140834, respectively. Another rabbit polyclonal antibody raised against GST-PIPKH was affinity-purified by passage over a glutathione-agarose-GST-PIPKH column, with the antibody eluted by acid.

Coexpression with PIPKs Iα, Iβ, and Iγ—293t HEK cells were cotransfected on 10-cm dishes with 2 μg of FLAG-PIPKH and 4 μg of HA-PIPK Iα, Iβ, or Iγ. PIPKH was immunoprecipitated with M2-agarose (anti-FLAG) beads, and the immunoprecipitate was analyzed by Western blotting using monoclonal anti-HA (HA11) from Berkeley Antibody Co.

In Vivo Labeling and Measurement of Phosphoinositides—293t cells were labeled at 37 °C with [^{3}H]inositol (10 μCi/ml) for 24 h in inositol-free Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum as described previously (10). Lipids were extracted in acid methanol followed by chloroform, and deacylated as described previously (31). Deacylated lipids were analyzed by HPLC using a shallow ammonium phosphate gradient (10–35 mM ammonium phosphate, pH 3.8) over 100 min, to separate the glycerol-phosphoryl-inositol phosphate derivatives of the cellular phosphoinositides (10).

Coexpression with GFP-tagged PH Domains—FLAG-tagged PIPKH or PIPKIβ (2 μg of DNA per well) and EGFP-tagged PH domains from Bruton's tyrosine kinase (BTK; 1 μg DNA per well) and phospholipase Cδ were cotransfected into NIH-3T3 cells to study the localization of PI(4,5)P_2 and PI(3,4,5)P_3, respectively, in serum-stimulated cells in the presence or absence of wortmannin. NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Cells grown on glass coverslips were transfected with pBTK-PH-EGFP, pdsRed1-N1, pFLAG-CMV2, pFLAG-CMV2-PIPKH, pCMV, and/or pCMV-PIPKH using LipofectAMINE Plus (Invitrogen) and observed 24 h after transfection. Wortmannin was used at a final concentration of 200 nM for 2 h prior to fluorescence microscopy. Live cells were imaged in a 37 °C microscope chamber using a Nikon Diaphot 300 microscope equipped with a Sensys CCD (charge-coupled device) digital camera (Photometrics), and images were processed using ImagePro Plus 4.1 (MediaCybernetics) and Vaytek Imaging Software (Fairfield, IA). Standard epifluorescence filter sets for EGFP and dsRed were obtained from Chroma Technologies. Single color control experiments demonstrated insignificant signal cross-contamination between channels. All images were captured in gray scale. Pseudocolor was added to allow comparisons between images when they were merged. Digital confocal images were obtained by imaging adjacent focal planes at 0.3-μm intervals, and three adjacent images underwent nearest-neighbor rapid deconvolution using a calculated point-spread function and Vaytek Microtome image deconvolution software.

Visualization of Actin in PIPKH-expressing Cells—NIH-3T3 cells were plated on coverslips in 6-well tissue culture plates and transfected with 0–4 μg of pFLAG-CMV2-PIPKH or pFLAG-CMV2-PIPKH ($155^{Lys\rightarrow Arg}$/$281^{Asp\rightarrow Ala}$), using LipofectAMINE PLUS reagent. After 24 h, cells were fixed for 15 min at room temperature in cytoskeletal buffer (CSB: 10 mM MES pH 6.1, 138 mM KCl, 3 mM $MgCl_2$, 2 mM EGTA) containing 3.7% paraformaldehyde. The cells were washed three times in CSB and permeabilized in CSB containing 0.1% Triton X-100 and 5% nonfat dry milk (NFDM). The coverslips were washed once in PBS, 0.1% Triton X-100, 5% NFDM and incubated with mouse monoclonal M5 anti-FLAG antibody in PBS, 0.1% Triton X-100, 5% NFDM for 1 h at 37 °C. The coverslips were washed three times in PBS, 0.1% Triton X-100, 5% NFDM, and incubated with CY2-conjugated goat anti-mouse secondary antibody plus rhodamine phalloidin (10 μg/ml) for 1 h at room temperature. The coverslips were mounted onto glass

slides with Fluoromount after three washes in PBS. Cells were visualized by epifluorescence and spinning disc confocal microscopy.

RESULTS

Identification of a Novel PIPK Homolog in the Human Genome Sequence Data Base—The sequence DYSLL, conserved in the lipid kinase domain catalytic core of all known PIPKs, was used to search the high throughput genome sequence data base to identify related sequences in the tBlastn search mode. As expected, the search identified all heretofore cloned PIPK genes (types I and II). In addition, a highly related gene sequence (clone AL157935) was identified on human chromosome 9. The cDNA sequence predicted to encode the putative chromosome 9 PIPK was assembled from the ENSEMBL data base and was used to identify related sequences in the nonredundant and EST nucleotide data bases in the Blastn search mode. ESTs corresponding to rat and mouse isoforms of the putative human chromosome 9 PIPK were identified and used to search the nucleotide data bases in the Blastn search mode. Numerous cDNA sequences were identified in this fashion in the RIKEN data base, from which three mouse clones, designated A330090A02, A330097I22, and A830027C23 (GenBank™ accession numbers BB195634, BB197130, and BB266750, respectively) were selected and generously provided by the RIKEN Tsukuba Institute (Genome Exploration Group, RIKEN Genomic Science Center, Genome Science Laboratory). The three clones were of identical sequence, encoding a protein of 395 amino acids, and all further analysis was performed using clone A330090A02. Fig. 1 displays the human and mouse isoforms aligned with human types Iα, Iβ, IIα, and IIβ PIPKs. The human sequence assembled from the genome data base using ENSEMBL predicts a protein containing an 83-residue N-terminal extension not found in the murine isoform. Two in-frame translation termination codons are present upstream of the putative initiation codon of the murine isoenzyme. The predicted molecular mass of the murine PIPKH is 45290 daltons. The primary sequence does not contain any known functional protein domain outside the catalytic (lipid kinase; residues 129–395) core, as analyzed by Pfam.

Tissue Distribution—At the level of mRNA, expression of PIPKH is highest in brain and testis relative to heart, spleen, lung, liver, skeletal muscle, and kidney, in which mRNA expression appears very low (Fig. 2) or absent. Multiple bands in the brain and testis lanes suggest the presence of multiple size variants of the mature message and alternative splicing of the primary transcript. However, we did not encounter splice variants in the process of sequencing multiple cDNA clones, both murine and human, of PIPKH. Western analysis of brain extracts (Fig. 3) demonstrates the presence in rat brain of a single molecular mass species of protein cross-reacting with an affinity-purified rabbit polyclonal antibody raised against GST-PIPKH. Analysis of mouse brain extracts yielded identical results (not shown). The human isoform, present in brain homogenates or expressed as a recombinant FLAG epitope-tagged protein in 293t cells, is only weakly cross-reactive with the antibody raised against murine PIPKH (not shown). The protein found in human brain is identical to the rat brain isoform in molecular mass (not shown), suggesting that the N-terminal extension of PIPKH predicted by the annotated human genome sequence is not actually expressed *in vivo* and that the actual N terminus is Met^{84} of the human isoform in Fig. 1. The endogenous rat brain protein shown in *lanes 4–6* of Fig. 5 appears to migrate slightly slower than does recombinant murine FLAG-PIPKH expressed in 293t human embryonic kidney cells, raising the possibility of tissue- or species-specific post-translational modification.

The Protein Product of PIPKH Clone A330090A02 Is Associated with PI(4)P 5-Kinase Activity When Expressed in 293t Cells but Not in Bacteria—Clone A330090A02 was expressed as N-terminal FLAG or hemagglutinin epitope-tagged (FLAG-PIPKH or HA-PIPKH) and GST fusion (GST-PIPKH) proteins in 293t human embryonic kidney cells and *Escherichia coli*, respectively. FLAG-PIPKH, HA-PIPKH, and GST-PIPKH were purified with anti-FLAG (M2)-, anti HA (HA11)-, and glutathione-coupled agarose beads, respectively, and assayed for phosphoinositide kinase activity (Fig. 4). The GST fusion protein expressed in bacteria and purified with glutathione-Sepharose beads displayed no detectable PI(4)P 5-kinase or PI(5)P 4-kinase activity (Fig. 4*a* and data not shown), whereas all type I PIPKs are enzymatically active when expressed in bacteria.[2] In contrast, when presented with crude brain phosphoinositides (not shown) or synthetic PI(4)P plus [γ-^{32}P]ATP, immunoprecipitates of FLAG epitope-tagged PIPKH expressed in 293t cells catalyzed ^{32}P incorporation into two lipids that comigrated with PI(4,5)P_2 and PI(3,4,5)P_3 standards by TLC (Fig. 4*a*). Deacylation and HPLC analysis of the two lipid products of the FLAG-PIPKH kinase reaction recovered from the TLC plates in both cases yielded radioactive species that comigrated with glycerol-phosphorylinositol 4,5-bisphosphate, indicating that the ^{32}P-labeled phosphoinositide comigrating with PI(3,4,5)P_3 is lyso-PI(4,5)P_2 and not PI(3,4,5)P_3. Supporting this conclusion was the detection of lyso-PI(4)P in the synthetic PI(4)P preparation, in a proportion to the diacyl form consistent with the relative activities of the two lipid products recovered from the TLC plates (data not shown). The PI(4)P 5-kinase activity associated with PIPKH was not inhibited by wortmannin (data not shown). Immunoprecipitates of FLAG epitope-tagged PIPKH exhibited no detectable activity on the following synthetic lipids: diacylglycerol, phosphatidic acid, PI(3)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, and PI(4,5)P_2 (Fig. 4*b* and data not shown). When similar amounts of recombinant protein were assayed for PI(4)P 5-kinase activity, immunoprecipitates of HA epitope-tagged PIPKH were found to exhibit relatively low activity, similar to that of catalytically inactivated mutants of PIPKIα and PIPKIβ, when compared with wild-type recombinant PIPKIα and PIPKIβ (Fig. 4*c*). When assayed for PI(5)P 4-kinase activity, immunoprecipitates of wild-type recombinant type I PIPKs catalyzed the formation of a small amount of ^{32}P-labeled lipid comigrating with PI(4,5)P_2 (Fig. 4*c*). This could be due to the presence either of contaminating PI(4)P in the PI(5)P substrate preparation or of type II PIPK in immunoprecipitates of type I PIPKs. In contrast, PIPKH and catalytically inactive mutants of type I PIPKs yielded no detectable product when PI(5)P was used as substrate (Fig. 4*c*). Thus, PI(4)P and lyso-PI(4)P appear to be the only phosphoinositide substrates of the catalytic activity associated with PIPKH.

PIPKH Does Not Bind to Immobilized Phospholipids in an in Vitro Transcription/Translation Binding Assay—We expressed pFLAG-CMV2-PIPKH in a rabbit reticulocyte lysate transcription/translation system incorporating [^{35}S]methionine. When *in vitro* translated PIPKH was applied to a PVDF membrane on which various phospholipids were arrayed, we did not detect significant binding to any of these lipids (data not shown), which included PI, PI(3)P, PI(4)P, PI(5)P, PI(3,4)P_2, PI(3,5)P_2, PI(4,5)P_2, PI(3,4,5)P_3, phosphatidic acid, phosphatidylcholine, and phosphatidylserine. As a positive control in the same experiment, *in vitro* translated AKT (expressed at a similar level) bound to PI(3,4)P_2 and PI(3,4,5)P_3.

Mutational Analysis of PIPKH Suggests That PI(4)P 5-Kinase Activity Is Not Intrinsic—Mutation of Lys^{155} to Arg or of

[2] J. D. Chang, unpublished observations.

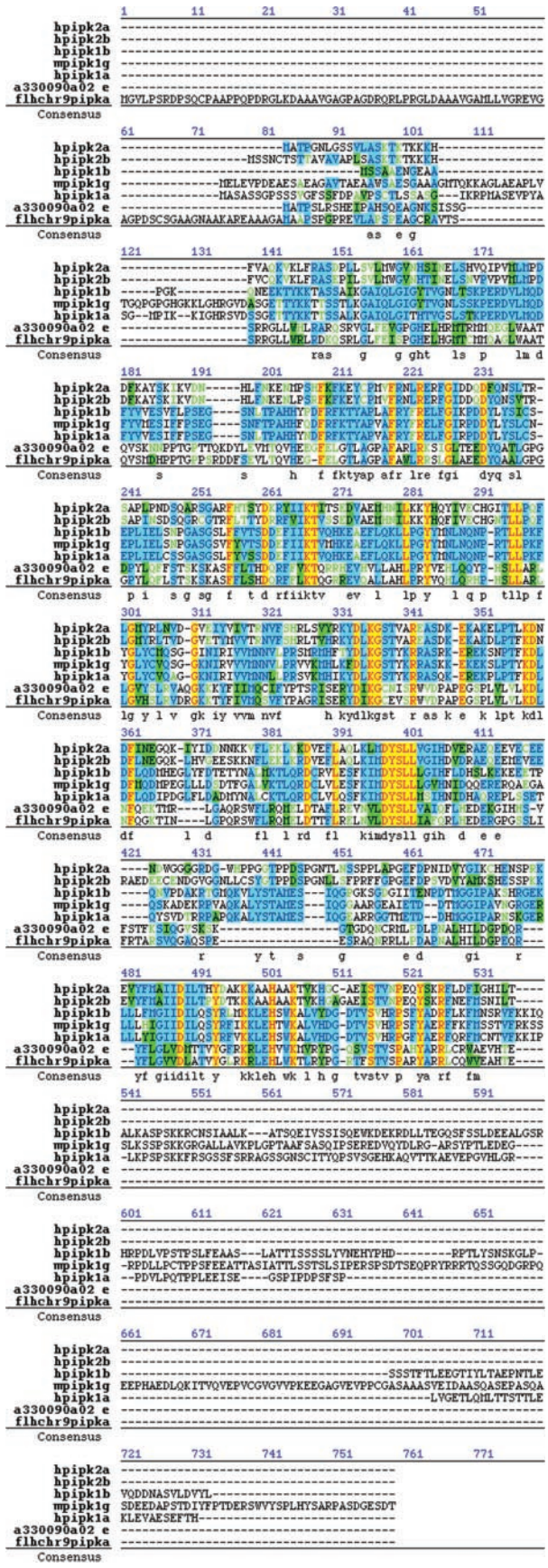

FIG. 1. **PIPKH amino acid sequence alignment.** Murine PIPKH (clone A330090A02) was aligned with the human sequence (flhchr9pipk) deduced from the high throughput genome data base; alignment includes sequences of human types IIα(hpipk2a), IIβ(hpipk2b), Iα(hpipk1a), and murine type Iγ(mpipk1g) PIPKIs. Amino acid residues highlighted in *yellow* are conserved in all PIPKs.

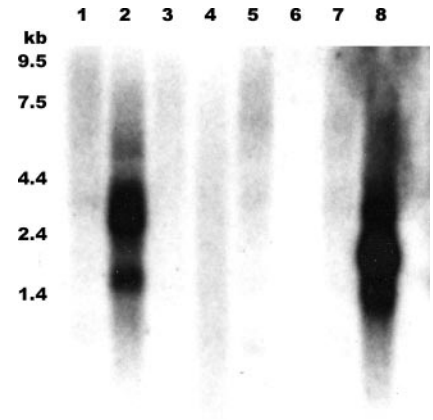

FIG. 2. **Northern analysis of PIPKH tissue distribution.** Multiple tissue Northern blot (Clontech) prepared with murine tissue RNA was probed with the entire coding region of murine PIPKH and exposed for 72 h at −80 °C. Heart, *lane 1*; brain, *lane 2*; spleen, *lane 3*; lung, *lane 4*; liver, *lane 5*; skeletal muscle, *lane 6*; kidney, *lane 7*; and testis, *lane 8*.

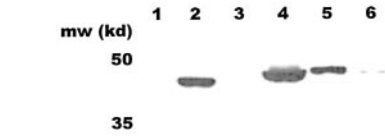

FIG. 3. **Western analysis of PIPKH in brain extracts.** Control 293t cell lysate, *lane 1*; lysate of 293t cells expressing recombinant murine FLAG-PIPKH, *lane 2*; lysate of 293t cells expressing recombinant human FLAG-PIPKH, *lane 3*; unfractionated rat brain homogenate, *lane 4*; high salt extract of rat brain membrane, *lane 5*; Triton X-100 extract of salt-extracted rat brain membrane, *lane 6*.

Asp^{281} to Ala (amino acids corresponding to PIPKH residues Lys^{155} and Asp^{281} are required for PIPK activity in type I PIPKs) resulted in no significant reduction in the level of lipid kinase activity associated with PIPKH; likewise, the PI(4)P 5-kinase activity associated with a double mutant (155$^{\text{Lys}\rightarrow\text{Arg}}$/281$^{\text{Asp}\rightarrow\text{Ala}}$) of PIPKH was similar to that of wild-type PIPKH (Fig. 4*a*).

PIPKH Associates with PIPKs Iα and Iβ—Since the PI(4)P 5-kinase activity in PIPKH immunoprecipitates did not appear to be intrinsic to the PIPKH itself, we investigated the possibility that PIPKH associates with an endogenous type I PIPK. Western analysis of lysates of 293t cells coexpressing FLAG-tagged PIPKH and HA-tagged PIPKIα or HA-PIPKIβ demonstrated a specific association between PIPKH and the other PIPKs (Fig. 5). When PIPKIγ was tested in this system, it was found to bind nonspecifically (*lanes 6* and *7*) to the anti-FLAG-agarose beads used to immunoprecipitate the FLAG-PIPKH complexes; therefore, although PIPKH may also associate with PIPKIγ *in vivo* (both are highly expressed in brain), we were unable to test for this association. High levels of background signal associated with available type I PIPK antibodies, and the fact that our PIPKH antibody does not immunoprecipitate, made it difficult to demonstrate unequivocally an association between endogenous PIPKH and the other PIPKs.

Expression of PIPKH in 293t Cells Results in Greatly Increased PI(3,4,5)P_3 Levels—Despite its apparent lack of intrinsic phosphoinositide kinase activity, and to determine the biological significance of its ability to associate with and potentially regulate active type I PIPKs, we measured total cellular phosphoinositide levels in cells expressing PIPKH. Transfection of [^{3}H]inositol-labeled 293t cells with pFLAG-CMV2-PIPKH resulted in an 8-fold increase, relative to cells transfected with empty vector, in the level of PI(3,4,5)P_3 measured by HPLC analysis of the deacylated cellular phospholipids

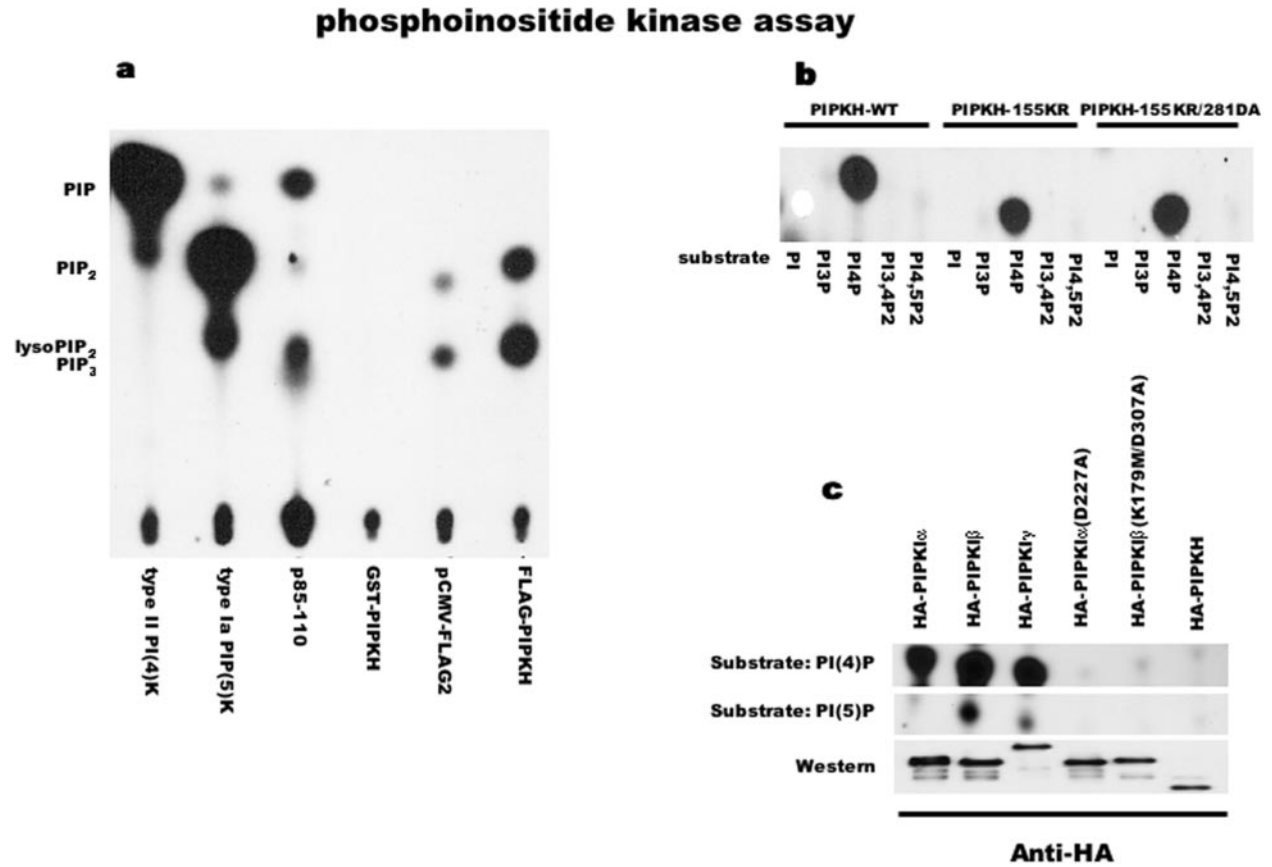

FIG. 4. **Phosphoinositide kinase assay.** *a*, long exposure (24 h at −80 °C) autoradiogram of thin layer chromatogram of lipid kinase reaction using crude brain phosphoinositide (type II PI(4)K and p85–110) or synthetic PI(4)P (type Iα PIPK, GST-PIPK, pFLAG-CMV2, and FLAG-PIPKH) as substrate. *b*, long exposure (24 h at −80 °C) autoradiogram of thin layer chromatogram of immunoprecipitation lipid kinase reaction using various phosphoinositide substrates as indicated. Wild-type PIPKH was compared with mutant $155^{Lys\rightarrow Arg}$ and double mutant $155^{Lys\rightarrow Arg}/281^{Asp\rightarrow Ala}$. PIPKH and mutants were immunoprecipitated with M2 (anti-FLAG)-agarose beads. *c*, short exposure (3 h at room temperature) autoradiogram of thin layer chromatogram of immunoprecipitation lipid kinase assay employing HA-tagged PIPKs and PIPK mutants expressed in 293t cells. The *top panels* show results when PI(4)P was employed as substrate; the *middle panels* show results when PI(5)P was employed as substrate; the *bottom panels* show immunoblot analysis of the input immunoprecipitate for each lipid kinase reaction.

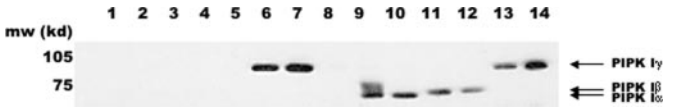

FIG. 5. **Coimmunoprecipitation; murine FLAG-PIPKH (*lanes 8–14*) or pCMV-FLAG2 vector (*lanes 1–7*) cotransfected with pEBB vector (*lanes 1* and *8*), HA-PIPK Iα (*lanes 2, 3, 9,* and *10*), HA-PIPK Iβ (*lanes 4, 5, 11,* and *12*), or HA-PIPK Iγ (*lanes 6, 7, 13,* and *14*).** Immunoprecipitation was with M2 (anti-FLAG)-agarose, and immunoblotting was with H11 anti-HA monoclonal antibody.

(Fig. 6). Because the transfection efficiency of pFLAG-CMV2-PIPKH in control 293t cell cultures ranged between 15 and 40% (data not shown), it is likely that the true -fold increase of $PI(3,4,5)P_3$ levels in individual cells actually expressing PIPKH was much higher than the 8-fold increase observed when the total population of transfected and untransfected 293t cells was extracted for phospholipid content. There was no net increase in the level of PI(4)P and $PI(4,5)P_2$ in these cells (Fig. 6). Expression of the double point mutant ($155^{Lys\rightarrow Arg}/281^{Asp\rightarrow Ala}$) of PIPKH also resulted in an elevation of $PI(3,4,5)P_3$ in 293t cells, although the increase was not as great as that seen in cells transfected with wild-type PIPKH. This could be due to an effect of the mutations on localization of PIPKH to membranes where PI(4)P substrate is available or on association of PIPKH with other proteins needed efficiently to convert $PI(4,5)P_2$ to $PI(3,4,5)P_3$.

Localization of Cellular Phosphoinositides in NIH-3T3 Cells Overexpressing PIPKH—In view of the dramatic increase in $PI(3,4,5)P_3$ levels in cells expressing PIPKH, we examined the effect of PIPKH expression on localization of cellular $PI(4,5)P_2$ and $PI(3,4,5)P_3$ by coexpressing in NIH-3T3 fibroblasts PIPKH and EGFP fusion proteins containing phosphoinositide-specific pleckstrin homology (PH) domains. When coexpressed with FLAG-PIPKH, EGFP-PLCδ-PH (specific for $PI(4,5)P_2$) displayed a plasma membrane-binding pattern (Fig. 7*A*), which was indistinguishable from the pattern observed when cells were cotransfected with empty vector (pFLAG-CMV2). However, when coexpressed with FLAG-PIPKH, EGFP-BTK-PH, a marker for $PI(3,4,5)P_3$, localized to large cytoplasmic vesicular structures (Fig. 7*B*). The pattern of EGFP-BTK-PH localization was similar in cells expressing the untagged version of PIPKH, whereas the $280^{Arg\rightarrow Cys}$ mutation in the PH domain of BTK, which abolishes its ability to bind $PI(3,4,5)P_3$, resulted in complete loss of the cytoplasmic vesicular pattern of localization observed with the wild-type BTK PH domain (data not shown), demonstrating that localization of EGFP-BTK-PH in cells expressing FLAG-PIPKH is due to interaction between PIPKH and $PI(3,4,5)P_3$. Most interesting, treatment of cells expressing PIPKH with 200 nM wortmannin (conditions that abolish EGFP-BTK-PH binding to plasma membranes in cells transfected with phosphatidylinositol 3-kinase p110C*AAX*) did not release EGFP-BTK-PH from the intracellular structures (Fig. 7*B*), suggesting that $PI(3,4,5)P_3$ turns over very slowly in this compartment.

Formation of Actin Comets and Foci in Cells Expressing PIPKH—When expressed in NIH-3T3 cells, FLAG-tagged

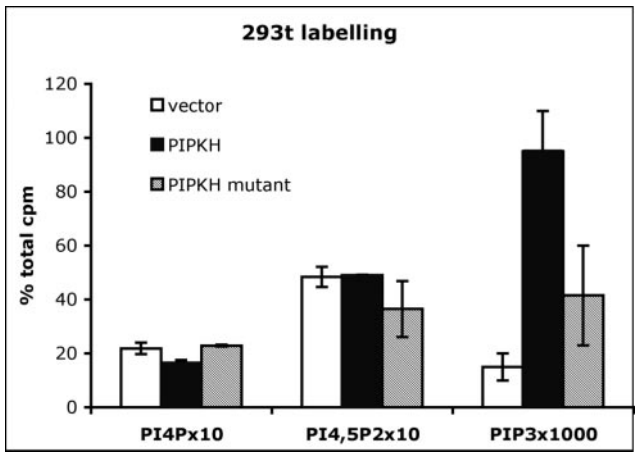

FIG. 6. **Measurement of total PI(4)P, PI(4,5)P_2, and PI(3,4,5)P_3 levels in 293t cells transfected with PIPKH or double point mutant PIPKH($155^{Lys\rightarrow Arg}/281^{Asp\rightarrow Ala}$).** 293t cells were transfected with pFLAG-CMV2-PIPKH, pFLAG-CMV2-PIPKH($155^{Lys\rightarrow Arg}/281^{Asp\rightarrow Ala}$), or empty vector (pFLAG-CMV2) and labeled with [^{3}H]inositol in inositol-free medium as described. Total cellular phospholipids were extracted, deacylated, and separated by HPLC. *Bars* indicate percentage of total ^{3}H cpm found in HPLC peaks corresponding to PI(4)P, PI(4,5,)P_2, and PI(3,4,5)P_3 after phosphoinositide extraction and deacylation. Values expressed in the *bar graph* represent data from four independent experiments.

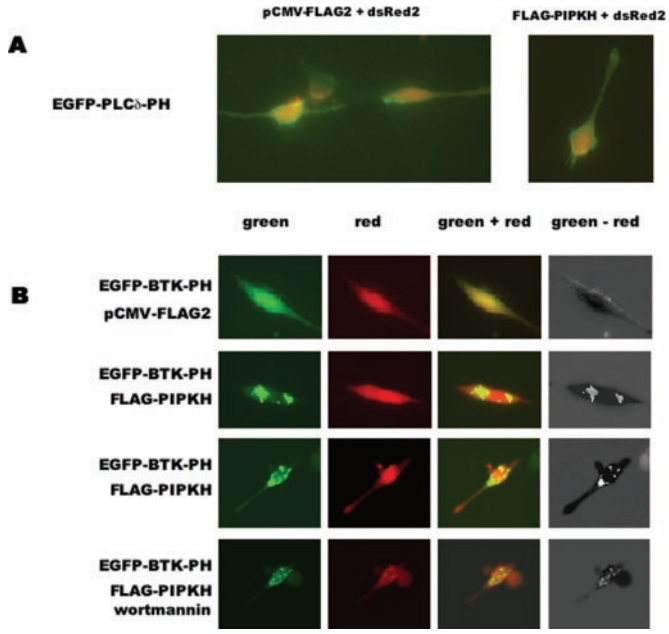

FIG. 7. **PI(4,5)P_2 and PI(3,4,5)P_3 localization in NIH-3T3 cells expressing FLAG-PIPKH.** NIH-3T3 cells were transfected with EGFP-tagged PH domain from phospholipase Cδ (*PLC*δ) (*A*) or Bruton's tyrosine kinase (*BTK*) (*B*) to visualize PI(4,5)P_2 or PI(3,4,5)P_3, respectively, together with pCMV-FLAG2 vector or FLAG-PIPKH as indicated, in the presence or absence of wortmannin 200 nM. pdsRed-N1 was included in the transfections to control for variability of cell volume.

PIPKH induced formation of actin comets and foci similar to those described in cells infected with *Listeria* or transfected with PIPKs Iα, β, or γ (16). Fig. 8 shows transfected cells stained with rhodamine-phalloidin and anti-FLAG-Cy2. All cells staining for the FLAG epitope-tagged PIPKH, as well as those expressing PIPKs Iα and Iβ (data not shown), displayed both comets as well as foci, with a marked reduction of stress fibers. Adjacent nontransfected cells showed mainly stress fibers, with only rare comets or foci. Comparison of Figs. 7 and 8 reveals a distinctly different pattern of localization for EGFP-BTK-PH and PIPKH. Whereas EGFP-BTK-PH appears to localize in large intracytoplasmic vesicular structures in cells expressing PIPKH, PIPKH itself appears to colocalize with the actin-based comets and foci (Fig. 8).

Cells expressing the PIPKH ($155^{Lys\rightarrow Arg}/281^{Asp\rightarrow Ala}$) mutant expressed fewer actin comets and foci than did those expressing the wild-type PIPKH (data not shown). In the former cells, organization of actin in stress fibers was better preserved than in the latter cells, in which most of the actin was reorganized into comets and foci.

DISCUSSION

Control over the multitude of processes involving membrane trafficking requires rapid, reversible, and localized assembly of the actin cytoskeleton, which confers upon these processes infrastructure, mechanical support, or directionality. Rapid and reversible assembly of the cytoskeletal framework for membrane vesicle formation, exocytosis, and cell migration is regulated by the generation of PI(4,5)P_2 by type I PIPKs. It is now well established that this phosphoinositide is a signaling molecule in its own right, acting through several actin-binding proteins and members of the WASP family of proteins, as well as by controlling the localization of a subset of proteins containing a PH domain. Localized generation of PI(4,5)P_2 marks membrane microdomains for specific structural and functional transformations, such as occur during endocytosis, exocytosis, cell migration, and cell adhesion. These transformations are effected by actin nucleation, clathrin coat assembly, and recruitment to specific membrane domains of scaffold and signaling proteins such as talin (4, 13) and dynamin (8, 9), all of which require PI(4,5)P_2. Further evidence that PI(4,5)P_2 regulates actin nucleation and polymerization comes from the observation of exuberant actin cytoskeletal growth at sites of synaptojanin 1 disruption (17, 18). Synaptojanin 1 is a polyphosphoinositide phosphatase concentrated at nerve terminals (19). The balance of PIPKIγ and synaptojanin 1 activity controls the levels of PI(4,5)P_2 at endocytic zones of synapses (14) and is responsible for the rapid and reversible nucleation of actin and recruitment of clathrin at nerve terminals. Presumably, a similar balance of PIPK/phosphatase activity exists in other cell types engaged in vesicle fission/fusion and membrane trafficking.

We have cloned a cDNA that encodes a novel protein that is homologous to the known PIPKs. When compared with type IIβ PIPK, whose crystal structure has elucidated its contact points with the inner leaflet of the cell membrane as well as with its lipid and ATP substrates (15), all amino acid residues predicted to participate in these interactions are conserved in PIPKH. However, the low level of lipid kinase activity detectable in immunoprecipitation kinase assays appears to be attributable to type I PIPKs Iα, Iβ, and possibly Iγ that coimmunoprecipitate with PIPKH. The high level of expression of PIPKIγ and PIPKH in brain may drive an association between these related proteins, although this could not be verified on the basis of our coimmunoprecipitation Western analysis. It is possible that the absence of activity associated with bacterially expressed PIPKH may reflect a requirement for a cofactor or post-translational modification *in vivo*. However, all of the known type I PIPKs are enzymatically active after bacterial expression,[2] whereas PIPKH is not. Our observation that PIPKs Iα and Iβ associate with PIPKH when coexpressed in 293t cells suggests that the effects of PIPKH in transfected cells are due to associated, rather than intrinsic, lipid kinase activity. Our observations of PI(3,4,5)P_3 accumulation in cytosolic vesicles and of actin comet/focus formation in NIH-3T3 cells expressing PIPKH suggest that this protein acts as a scaffold to localize

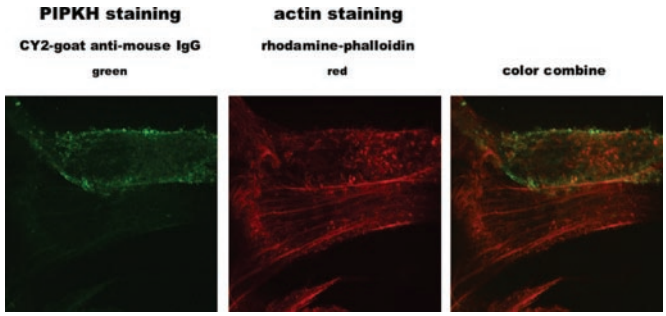

FIG. 8. **Formation of actin comets and foci in cells expressing FLAG-PIPKH.** NIH-3T3 cells were transfected with FLAG-PIPK and fixed 24 h after transfection. Actin was visualized by staining with rhodamine-conjugated phalloidin, and PIPKH was visualized by staining with M5 (anti-FLAG) monoclonal antibody, using CY2-conjugated goat anti-mouse Ig G secondary antibody. The cell in the *upper portion* of the figure is expressing FLAG-PIPKH, whereas the cell on the *bottom portion* does not express FLAG-PIPKH.

PIPK types Iα, Iβ, and possibly Iγ to specific compartments within the cell, where they generate PI(4,5)P_2 for actin nucleation, signaling, and scaffold protein recruitment and conversion to PI(3,4,5)P_3.

Type IIβ PIPK is known to exist as a homodimer in solution; the molecular interaction is mediated by antiparallel apposition of the β1-strand of a subunit-spanning β-sheet (15), as well as by packing of the α1-helix, forming a clasp-like interface. Types Iα and Iβ PIPKs are capable of forming homo- as well as heterodimers in pull-down experiments.[3] The sequence of murine PIPKH (positions 156–172 in Fig. 1) corresponding to the α1-helix of PIPKIIβ contains 6 (out of 17) residues that are conserved or conservatively substituted relative to PIPKIIβ, whereas the sequence of murine PIPKH (positions 189–194 in Fig. 1) corresponding to the β1-strand of PIPKIIβ does not share sequence similarity with any of the other PIPKs. It is therefore unclear whether the interaction we have observed between PIPKH and types Iα and Iβ PIPKs is mediated by the similar molecular contacts as demonstrated for the PIPK IIβ homodimer.

The foregoing observations are remarkably reminiscent of previously reported interactions between myotubularin, a dual-specificity protein-tyrosine and phosphoinositide 3-phosphatase, and a catalytically inactive homolog termed 3-phosphatase adaptor protein (3-PAP) (20–22). Myotubularin defines a large family of lipid phosphatases that includes eight catalytically active enzymes as well as six putative inactive homologs, of whose function only that of 3-PAP has been elucidated. Based on the purification of 3-PAP as a heterodimer with a PI(3)P 3-phosphatase (later identified as myotubularin), the presence of phosphoinositide 3-phosphatase activity in 3-PAP immunoprecipitates from platelet cytosol, the absence of phosphatase activity in recombinant 3-PAP expressed in insect cells, and the ability of 3-PAP to translocate myotubularin from plasma membrane to cytosol when coexpressed as recombinant epitope-tagged proteins in COS-7 cells (20–22), 3-PAP has been defined as a scaffold protein for the localization of catalytically active phosphoinositide phosphatases much in the same manner as we have done here for PIPKH and active phosphoinositide kinases.

In cells expressing PIPKH, we observed marked reorganization of actin from the stress fibers seen in untransfected NIH-3T3 cells to structures that resemble comet and focus formations described previously in cells infected with obligate intracellular pathogens such as *Listeria* and in cells transfected with active type I PIPKs (16). The regulation of PIPKs has been elucidated recently with the findings that Rac-1 and ARF family proteins associate or colocalize with PIPKIα and Iβ (reviewed in Ref. 2). Rac-1 interacts with PIPKs type Iα and Iβ via its C terminus. Rac-1-associated PIPK activity is not affected by whether the small G protein is in the GTP- or GDP-bound state, which implies that the role of Rac-1 in PIPK regulation has more to do with localization than with activation (6). We postulate that PIPKH may play a similar role. ARF family members, in particular ARF-6, colocalize with type I PIPKs and are known to be activators of phospholipase D. A product of phospholipase D, phosphatidic acid, has been demonstrated to stimulate type I PIPKs, leading to the hypothesis that ARFs stimulate PIPKs indirectly, by increasing production of an allosteric effector molecule and not by any direct interaction (23). Type I PIPKs are highly active when expressed as bacterial GST fusion proteins and do not seem to require any cofactor for enhancement of activity when measured in a purified preparation. This implies that regulation of the biological function of type I PIPKs is mainly by localization.

In light of the similar amounts of associated PIPK activity measured in immunoprecipitates of wild-type and point mutant PIPKH, we were surprised to find not only less PI(3,4,5)P_3 produced in 293t cells expressing the mutant PIPKH but also less dramatic reorganization of actin from stress fibers to comets/foci. Trivial explanations for this observation might include the following: 1) lower transfection efficiency or expression levels for the mutant PIPKH construct; 2) aberrant folding of the mutant PIPKH protein leading to impairment of one or more of the intermolecular associations responsible for PI(3,4,5)P_3 production and actin cytoskeletal rearrangement. However, on the basis of both Western analysis of FLAG epitope-tagged PIPKH and its mutant forms described above as well as immunoprecipitation phosphoinositide kinase assays (Fig. 4*b*), it appears that the mutant forms of PIPKH do express at similar levels and associate equally well with active type I PIPKs. We are now investigating other possibilities that might explain our observations on the mutant PIPKH.

A notion that has become increasingly well supported by experimental observations during the past several years is that PI(4,5)P_2-mediated signaling may be preferentially localized to membrane microdomains, known as caveolae or rafts, that are rich in cholesterol and sphingolipids (24). Rozelle *et al.* (16) demonstrated that all three of the known type I PIPKs, when expressed in REF52 cells, induce formation of filamentous actin comets as well as globular actin foci in a manner dependent on functional WASP proteins and Arp2/3 complex. WASP and related Scar proteins potentiate nucleation and branching (dendritic nucleation) of actin filaments by Arp2/3 complex (7). Inhibition of actin comet formation by depletion of membrane cholesterol suggested that sphingolipid/cholesterol-rich rafts may be the favored platform for PI(4,5)P_2-mediated signaling. The reported localization of type II PI(4)-kinase (25, 26) to synaptic vesicles (27), endosomes (28), and Golgi (29), all of which are associated with raft formations, implies that rapid, coupled phosphorylation of PI and PI(4)P takes place in these structures to generate PI(4,5)P_2 required for the process of membrane trafficking.

A model that might explain our and others' observations in cells overexpressing PIPKs or PIPKH is that the dramatic reorganization of actin, from filamentous structures such as stress fibers to globular structures such as comets and foci, is due to a unique spatial distribution of PI(4,5)P_2 and/or PI(3,4,5)P_3 produced in these cells. The PI(4,5)P_2 and/or PI(3,4,5)P_3 formed as a result of PIPK/PIPKH overexpression may preferentially potentiate multiple dendritic nucleation

[3] C. L. Carpenter, unpublished data.

events, leading to a bias toward radial, as opposed to linear, growth of the actin polymer. Although actin comets are rarely visualized in mammalian cells (16), similar structures are found in cells infected with intracellular pathogens such as *Listeria* and *Shigella*, which employ mechanical force generated by actin polymerization to propel themselves around the cytoplasm. ActA, a protein found on the cell surface of such pathogens, appears to serve a role analogous to WASP/Scar proteins, namely to potentiate actin nucleation and branching by Arp2/3 complex. Actin comet formation in cells overexpressing PIPKs or PIPKH might then be interpreted as resulting from a process of Arp2/3 complex activation similar to that initiated by ActA in *Listeria*-infected cells.

The marked increase in total cellular $PI(3,4,5)P_3$ as well as its unusual localization in intracytoplasmic vesicles in cells overexpressing PIPKH were unexpected observations, given the absence of $PI(4,5)P_2$ elevation in these cells. One possible explanation for our findings is that conversion of $PI(4,5)P_2$ to $PI(3,4,5)P_3$ in cells expressing PIPKH is rapid and takes place in a cellular compartment deficient in phosphoinositide phosphatase activity. We have observed that there is a pool of PI(3)P that appears to be resistant to dephosphorylation when cells are treated with concentrations of wortmannin sufficient to inhibit completely all phosphatidylinositol 3-kinase activity.[2] We plan to investigate the possibility that PIPKH acts as a scaffold to bring type I PIPKs in proximity with other proteins necessary for efficient production of $PI(3,4,5)P_3$.

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REFERENCES

1. Ma, L., Cantley, L. C., Janmey, P. A., and Kirschner, M. W. (1998) *J. Cell Biol.* **140,** 1125–1136
2. Hinchliffe, K. (2000) *Curr. Biol.* **10,** R104–R105
3. De Camilli, P., Emr, S. D., McPherson, P. S., and Novick, P. (1996) *Science* **271,** 1533–1539
4. Ling, K., Doughman, R. L., Firestone, A. J., Bunce, M. W., and Anderson, R. A. (2002) *Nature* **420,** 89–93
5. Ishihara, H., Shibasaki, Y., Kizuki, N., Wada, T., Yazaki, Y., Asano, T., and Oka, Y. (1998) *J. Biol. Chem.* **273,** 8741–8748
6. Tolias, K. F., Hartwig, J. H., Ishihara, H., Shibasaki, Y., Cantley, L. C., and Carpenter, C. L. (2000) *Curr. Biol.* **10,** 153–156
7. Higgs, H. N., and Pollard, T. D. (1999) *J. Biol. Chem.* **274,** 32531–32534
8. Lee, E., and De Camilli, P. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 161–166
9. Orth, J. D., Krueger, E. W., Cao, H., and McNiven, M. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 167–172
10. Rameh, L. E., Tolias, K. F., Duckworth, B. C., and Cantley, L. C. (1997) *Nature* **390,** 192–196
11. Tolias, K., and Carpenter, C. L. (2000) *Methods Enzymol.* **325,** 190–200
12. Honda, A., Nogami, M., Yokozeki, T., Yamazaki, M., Nakamura, H., Watanabe, H., Kawamoto, K., Nakayama, K., Morris, A. J., Frohman, M. A., and Kanaho, Y. (1999) *Cell* **99,** 521–532
13. Di Paolo, G., Pellegrini, L., Letinic, K., Cestra, G., Zoncu, R., Voronov, S., Chang, S., Guo, J., Wenk, M. R., and De Camilli, P. (2002) *Nature* **420,** 85–89
14. Wenk, M. R., Pellegrini, L., Klenchin, V. A., Di Paolo, G., Chang, S., Daniell, L., Arioka, M., Martin, T. F., and De Camilli, P. (2001) *Neuron* **32,** 79–88
15. Rao, V. D., Misra, S., Boronenkov, I. V., Anderson, R. A., and Hurley, J. H. (1998) *Cell* **94,** 829–839
16. Rozelle, A. L., Machesky, L. M., Yamamoto, M., Driessens, M. H., Insall, R. H., Roth, M. G., Luby-Phelps, K., Marriott, G., Hall, A., and Yin, H. L. (2000) *Curr. Biol.* **10,** 311–320
17. Cremona, O., Di Paolo, G., Wenk, M. R., Luthi, A., Kim, W. T., Takei, K., Daniell, L., Nemoto, Y., Shears, S. B., Flavell, R. A., McCormick, D. A., and De Camilli, P. (1999) *Cell* **99,** 179–188
18. Gad, H., Ringstad, N., Low, P., Kjaerulff, O., Gustafsson, J., Wenk, M., Di Paolo, G., Nemoto, Y., Crun, J., Ellisman, M. H., De Camilli, P., Shupliakov, O., and Brodin, L. (2000) *Neuron* **27,** 301–312
19. McPherson, P. S., Garcia, E. P., Slepnev, V. I., David, C., Zhang, X., Grabs, D., Sossin, W. S., Bauerfeind, R., Nemoto, Y., and De Camilli, P. (1996) *Nature* **379,** 353–357
20. Nandurkar, H. H., Caldwell, K. K., Whisstock, J. C., Layton, M. J., Gaudet, E. A., Norris, F. A., Majerus, P. W., and Mitchell, C. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9499–9504
21. Nandurkar, H. H., and Huysmans, R. (2002) *IUBMB Life* **53,** 37–43
22. Nandurkar, H. H., Layton, M., Laporte, J., Selan, C., Corcoran, L., Caldwell, K. K., Mochizuki, Y., Majerus, P. W., and Mitchell, C. A. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 8660–8665
23. Fensome, A., Cunningham, E., Prosser, S., Tan, S. K., Swigart, P., Thomas, G., Hsuan, J., and Cockcroft, S. (1996) *Curr. Biol.* **6,** 730–738
24. Harder, T., and Simons, K. (1997) *Curr. Opin. Cell Biol.* **9,** 534–542
25. Minogue, S., Anderson, J. S., Waugh, M. G., dos Santos, M., Corless, S., Cramer, R., and Hsuan, J. J. (2001) *J. Biol. Chem.* **276,** 16635–16640
26. Barylko, B., Gerber, S. H., Binns, D. D., Grichine, N., Khvotchev, M., Sudhof, T. C., and Albanesi, J. P. (2001) *J. Biol. Chem.* **276,** 7705–7708
27. Guo, J., Wenk, M. R., Pellegrini, L., Onofri, F., Benfenati, F., and De Camilli, P. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 3995–4000
28. Balla, A., Tuymetova, G., Barshishat, M., Geiszt, M., and Balla, T. (2002) *J. Biol. Chem.* **277,** 20041–20050
29. Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., and Cantley, L. C. (2002) *Mol. Cell* **10,** 151–162
30. Tolias, K. F., Rameh, L. E., Ishihara, H., Shibasaki, Y., Chen, J., Prestwich, G. D., Cantley, L. C., and Carpenter, C. L. (1998) *J. Biol. Chem.* **273,** 18040–18046
31. Serunian, L. A., Auger, K. R., and Cantley, L. C. (1991) *Methods Enzymol.* **198,** 78–87