A Novel Phosphatidylinositol-5-phosphate 4-Kinase (Phosphatidylinositol-phosphate Kinase IIγ) Is Phosphorylated in the Endoplasmic Reticulum in Response to Mitogenic Signals*

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Here, we identify a novel rat phosphatidylinositol-5-phosphate 4-kinase, phosphatidylinositol-phosphate kinase IIγ (PIPKIIγ). PIPKIIγ comprises 420 amino acids with a molecular mass of 47,048 Da, showing greater homology to the type IIα and IIβ isoforms (61.1 and 63.7% amino acid identities, respectively) of phosphatidylinositol-phosphate kinase than to the type I isoforms. It is predominantly expressed in kidney, with low expression in almost all other tissues. PIPKIIγ was found to have phosphatidylinositol-5-phosphate 4-kinase activity as demonstrated in other type II kinases such as PIPKIα. The PIPKIIγ that is present endogenously in rat fibroblasts, PC12 cells, and rat whole brain lysate or that is exogenously overexpressed in COS-7 cells shows a doublet migrating pattern on SDS-polyacrylamide gel electrophoresis. Alkaline phosphatase treatment and metabolic labeling in [32P]orthophosphate experiments revealed that PIPKIIγ is phosphorylated in vivo, resulting in a shift in its electrophoretic mobility. Phosphorylation is induced by treatment of mitogens such as serum and epidermal growth factor. Immunostaining experiments and subcellular fractionation revealed that PIPKIIγ localizes dominantly in the endoplasmic reticulum (ER). Phosphorylation also occurs in the ER. Thus, PIPKIIγ may have an important role in the synthesis of phosphatidylinositol bisphosphate in the ER.

Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) is a phospholipid with a variety of functions in vivo including not only the production of second messengers such as diacylglycerol and inositol 1,4,5-trisphosphate, but also the regulation of actin regulatory proteins and the activation of phospholipase D and ADP-ribosylation factor. It has also been reported that PI(4,5)P2 synthesis is potentiated by various stimuli including GTPγS (1–3), phorbol esters (4), tyrosine kinases (5), and integrins (6). The variations in its function and the regulation of its synthesis indicate that enzymes responsible for the production of PI(4,5)P2, such as PI kinase and PIPK, also show large diversities. Among PIPKs, two major subtypes (types I and II), each comprising two isoforms (Iα, Iγ, IIα, and IIβ), have been identified to date (13, 16–18), and it is thought that the role for each subtype in vivo is different. The type I isoyme has been reported to be activated by phosphatidic acid (7), to bind physically to the small GTPases Rho (8) and Rac (9), and to be involved in Ca2+-dependent exocytosis in PC12 cells (10). Human PIPKIβ has been shown to be identical to the STIM1 gene, the putative gene responsible for Friedreich’s ataxia, suggesting that this isoyme plays roles in vesicular trafficking such as neurotransmitter release (11). On the other hand, type II isoymes have also been reported to have several functions in vivo. In platelets, PIPKIIα was shown to translocate to the cytoskeletal fraction after stimulation by thrombin (12). PIPKIβ was identified by its specific interaction with a cytoplasmic region of the p55 tumor necrosis factor-α receptor, and a role for PIPK in tumor necrosis factor-α signaling has been suggested (13).

Here, we identify a novel PIPKII isoyme (PIPKIIγ) by a reverse transcription-PCR method using degenerate primers designed from highly conserved primary sequences in PIPK family members. PIPKIIγ is phosphorylated on serine residues in vivo, resulting in a mobility shift on SDS-polyacrylamide gel electrophoresis. Mitogenic stimulation, such as by serum, EGF, or PDGF treatment, results in phosphorylation of PIPKIIγ. The results of immunofluorescence experiments and subcellular fractionation suggest that PIPKIIγ has important roles in the production of PI(4,5)P2 in the ER.

EXPERIMENTAL PROCEDURES

Materials—PIPs were purified by neomycin column chromatography from crude phospholipids extracted from bovine spinal cord as described (14). [γ-32P]ATP, [γ-32P]dCTP, [32P]orthophosphate, and [γ32P]Pi(4,5)P2 were from NEN Life Science Products. The Colony/PlateScreen used to screen the cDNA library was from NEN Life Science Products. The polyvinylidene difluoride membranes used for Western blot analysis were from Nihon Eido (Tokyo, Japan). Nf2-nitrotriatomic acid-agarose was from QIAGEN Inc. (Westchester, PA). The Partisphere SAX column was from Whatman International Ltd. (Maidstone, United Kingdom). The thin-layer chromatography silica plates and the cellulose plate used to separate phospholipids and phosphoamino acids, respectively, were from Merck (Darmstadt, Germany).

Monoclonal anti-Myc antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-BiP antibody was from Stressgen Biotech Corp. Monoclonal anti-β-tubulin antibody was from Chemicon International, Inc. (Temecula, CA). Rhodamine- and fluorescein-conjugated anti-rabbit IgG antibodies and fluorescein-conjugated anti-mouse IgG antibody were from Organon Teknika Corp. (West Chester, PA). Rhodamine-conjugated wheat germ agglutinin was from Molecular Probes, Inc. (Eugene, OR).
Cell Culture—COS-7 and 3Y1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. PC12 cells were grown in Dulbecco's modified Eagle's medium containing 10% horse serum and 5% fetal bovine serum.

Reverse Transcription-Polymerase Chain Reaction—Total RNA isolated from rat liver was reverse-transcribed into cDNA by murine leukemia virus reverse transcriptase and used as a template for PCR using degenerate primers (5'-GAYTAYGGYCCIWGGTTYM-3', 5'-ATCYIABIAHARRCTTTRCTCCT-3', and 5'-ATCYIABIAHARI-GARTCTTCACT-3') corresponding to two highly conserved sequences in mammalian and yeast PIPKs (D/E)YCPXVFR and MDYSLLLIG/V/M. The polymerase chain reaction was carried out as follows: 95°C for 1 min, 43°C for 1 min, and 72°C for 2 min, for 40 cycles. The PCR product, ~500 base pairs long, was subcloned into the Smal site of the pBluescript SK- vector and sequenced.

cDNA Cloning of PIPKII—The PCR product encoding a novel sequence was cut out from the vector with EcoRI and BamHI, labeled with [α-32P]dCTP, and used as a probe for screening a rat brain cDNA library. The longest clone obtained (~2.4 kilobases) encoded an open reading frame as long as ~300 amino acids, but did not include a potential start codon. On the other hand, another partial clone was obtained that included a potential start codon preceded by a sequence consistent with a Kozak consensus sequence (15), but did not include a stop codon. From the sequences of these two clones, we could determine the complete sequence for this novel PIPK.

Northern Blot Analysis—A partial fragment corresponding to 418–1500 base pairs of cDNA was labeled and used as a probe for Northern blot analysis. Hybridization was carried out on mouse multiple tissue Northern blot membrane (MTNM TM, CLONTECH).

Production of Polyclonal Antibody—A partial fragment encoding amino acids 130–420 was ligated into the PstI-HindIII site of a pQE32 His tag expression vector (QIAGEN Inc.). The His-tagged protein was expressed in Escherichia coli and purified on Ni2+–nitritotriacetic acid agarose as described by the manufacturer. The purified protein was injected as an antigen into rabbits to raise polyclonal antisera. The resulting antibody was affinity-purified with the antigen protein transduced by an insert domain showing no similarity to other PIPKII homologs, Mss4p and Fab1p. Total RNA isolated from rat brain was reverse-transcribed and used as a template for further PCR. The PCR product was subcloned into the pBluescript vector and sequenced. Among several sequences corresponding to known PIPKs, one novel sequence homologous to the type II isoform of PIPK was obtained. We then tried to isolate a full-length cDNA of this novel PIPK. To study the tissue distribution of PIPKII, Northern hybridization was carried out on a full-length cDNA of this novel PIPK.
mRNA from various mouse tissues. An mRNA of 3.5 kilo-bases was detected in almost all tissues, with the most abundant expression in kidney (Fig. 2). The pattern of distribution is different from that of any type I isoform or any other type II isoform (13, 16–18), suggesting specific functions for this isoform.

PIPKIIγ Is a Phosphatidylinositol-5-phosphate 4-Kinase—We transfected a Myc-tagged version of the full-length cDNA of PIPKIIγ into COS-7 cells. The protein expressed in the whole cell lysate and the anti-Myc immunoprecipitate was detected in a doublet form by Western blotting with anti-Myc antibody (Fig. 3A and discussed further below). Myc-PIPKIIγ was immunoprecipitated with anti-Myc antibody, and the PIPK activity was measured. The immunoprecipitate phosphorylated PIP purified from bovine spinal cord (see “Experimental Procedures”), whereas anti-Myc immunoprecipitates from cells transfected with vector alone failed to do so (Fig. 3B). By using SAX HPLC, the resulting PIP2 was confirmed to be PI(4,5)P2 (Fig. 3C).

Recently, Rameh et al. (21) showed that the PIPKIIα is a
PI5P 4-kinase and slightly different from a PI4P 5-kinase. To examine whether PIPKIIγ is a PIP 4- or 5-kinase, \[^{32}P\]PI(4,5)P\(_2\) produced from the mixture of PI4P and PI5P by PIPKIIγ was treated with SHIP, which has been reported to dephosphorylate preferentially position 5 of PI(4,5)P\(_2\) (21). Upon SHIP treatment, the \[^{32}P\]PI(4,5)P\(_2\) spot was decreased, whereas a \[^{32}P\]PIP spot appeared (Fig. 3D). This \[^{32}P\]PIP was confirmed to be \[^{32}P\]PI(4)P by SAX HPLC (Fig. 3E). The same \[^{32}P\]PI(4)P spot was also detected when \[^{32}P\]PI(4,5)P\(_2\) produced by PIPKIIβ was used (Fig. 3D). On the other hand, \[^{32}P\]PI(4,5)P\(_2\) produced by PIPKIIβ did not show any \[^{32}P\]PI(4)P spot visible in a short exposure. But the long exposure showed a \[^{32}P\]PIP spot (Fig. 3D), which might represent \[^{32}P\]PI(4)P produced by a weak 4-phosphatase activity of SHIP (21). The identification of the \[^{32}P\]PI(4)P spot was also carried out by SAX HPLC (Fig. 3E). These results indicate that PIPKIIγ is a PI5P 4-kinase, like other type II isoforms.

**PIP KIIγ Is a Phosphoprotein**—A polyclonal antibody was produced with a partial His-tagged protein expressed in *E. coli* as an antigen. With this polyclonal antibody, endogenous PIPKIIγ was detected as doublet bands at 47 kDa in lysates from rat brain, PC12 cells, and 3Y1 fibroblasts by Western blotting (Fig. 4A). When the full-length cDNA (without the Myc tag) was transfected into COS-7 cells, the same doublet band was detected (Fig. 4A), suggesting that this doublet corresponds to some modification of PIPKIIγ such as proteolysis or phosphorylation and is not due to cross-reactivity of the antibody to another protein. To examine the possibility that these doublet bands correspond to phosphorylated PIPKIIγ, we treated the Myc-tagged version of PIPKIIγ with alkaline phosphatase. The Myc-PIP KIIγ that was immunoprecipitated from overexpressing COS-7 cells showed a doublet banding pattern with the upper band predominant. When the immunoprecipitated Myc-PIP KIIγ was incubated with calf intestine alkaline phosphatase, the upper band disappeared completely (Fig. 4B), whereas the lower band increased in intensity. This indicates that the doublet migrating pattern is due to the phosphorylation of PIPKIIγ. To confirm this conclusion, we next labeled PC12 cells metabolically with \[^{32}P\]orthophosphate. After labeling, the cells were lysed, and PIPKIIγ was immunoprecipitated, showing that PIPKIIγ was phosphorylated (Fig. 4C). Together with the results of Western blotting, it was confirmed that this phosphorylated protein corresponds to the upper band of PIPKIIγ (Fig. 4C). Next, the phosphorylated band was cut out from membrane, and phosphoamino acid analysis was carried out. The results show that PIPKIIγ phosphorylation occurs predominantly on serine residues (Fig. 4D). To determine whether the enzymatic activity of PIPKIIγ is affected by its phosphorylation, we measured the activity of Myc-PIP KIIγ after alkaline phosphatase treatment. Myc-PIP KIIγ retained considerable activity even after alkaline phosphatase treatment (Fig. 4E), indicating that the phosphorylation of PIPKIIγ does not affect its enzymatic activity.

**PIP KIIγ Is Phosphorylated in Response to Extracellular Stimuli**—In response to extracellular stimuli such as growth factors or hormones, intracellular protein kinases are activated and phosphorylate their physiological substrates. Since PIPKIIγ was found to be phosphorylated on serine residues in vivo, we examined whether the level of PIPKIIγ phosphorylation is potentiated by extracellular stimuli. First, we treated rat 3Y1 fibroblasts with 10% serum for various periods. The upper band of PIPKIIγ increased in a time-dependent manner, suggesting that PIPKIIγ is phosphorylated in response to serum (Fig. 5A). We then examined other extracellular stimuli including EGF, PDGF, bradykinin, and lysophosphatidic acid for their abilities to induce the phosphorylation of PIPKIIγ. Among them, EGF and PDGF enhanced the phosphorylation as well as serum (Fig. 5, B and C). Lysophosphatidic acid and bradykinin also induced phosphorylation to a lesser extent. Fig. 4D clearly shows that PIPKIIγ phosphorylation does not take place on tyrosine residues. Moreover, PIPKIIγ was not recognized by an anti-phosphotyrosine antibody, PY20 (data not shown). Therefore, it seems likely that the phosphorylation is mediated by a serine/threonine kinase downstream of mitogenic signals mediated by receptor tyrosine kinases. Protein kinase C does not seem to be involved since phorbol 12-myristate 13-acetate did not potentiate phosphorylation (Fig. 5, B and C). In addition, a specific protein kinase C inhibitor, H-7, did not suppress phosphorylation in 3Y1 cells (data not shown).

**Intracellular Localization of PIPKIIγ**—Using a polyclonal antibody, we next examined the intracellular localization of PIPKIIγ. The polyclonal antibody used was confirmed to recognize specifically the doublet band corresponding to PIPKIIγ in 3Y1 cell lysates by Western blotting (Fig. 4A). When rat 3Y1 fibroblasts were stained, PIP KIIγ was seen to predominate in the perinuclear regions, suggesting that it is localized in microsomal organelles such as the ER. To confirm this possibility, we double-stained rat 3Y1 fibroblasts with anti-PIP KIIγ antibody and with anti-BiP antibody, an ER-retaining protein. Both staining patterns (Fig. 6A) clearly indicate the localization of this enzyme in the ER. This staining pattern does not overlap with that of wheat germ agglutinin, a trans-Golgi staining reagent (Fig. 6A).

The intracellular localization of PIPKIIγ was studied further by a subcellular fractionation method using a 1.3 M sucrose cushion (see “Experimental Procedures”). PIPKIIγ was detected in smooth and rough microsomal fractions of rat liver the same as the ER marker BiP, whereas a cytosolic protein, β-tubulin, was detected only in the top of the gradient (Fig. 6B). The phosphorylated form of PIPKIIγ was also detected in these fractions, suggesting that phosphorylation of PIPKIIγ occurred in microsomes. To confirm this possibility, post-mitochondrial supernatants of 3Y1 fibroblasts were subjected to the same subcellular fractionation after mitogenic stimulations. PIPKIIγ, together with BiP, was detected predominantly in the smooth microsomal fraction (Fig. 6C). On stimulation by EGF, the phosphorylated form increased in this fraction, indicating that the phosphorylation of PIPKIIγ occurred within the ER (Fig. 6C). In addition, immuno-
fluorescence staining also showed that the localization of PIPKIIγ in the ER was not affected by stimulation of the cells with serum or EGF (data not shown). These results indicate that PIPKIIγ is phosphorylated in the ER in response to mitogenic signals, thus suggesting that it has important roles in the synthesis of PIP2 in the ER.

**DISCUSSION**

Purification and cDNA cloning of the 53-kDa PIPKIIα from erythrocytes revealed that the lipid kinase belongs to a distinct
lysates from rat brain, PC12 cells, 3Y1 cells, and PIPKIIα/presumably Myc-tagged PIPKIIα isoform and named it PIPKIIα. However, since we sequenced the cDNA of PIPKIIα, we succeeded in identifying a novel PIPK family different from those of PI 3- and PI 4-kinases and protein kinases (16). This family also seems to include yeast homologs such as Mss4p and Fab1p. Furthermore, cDNA cloning of types Iα and Iβ, members of another subtype of mammalian PIPK, also showed them to belong to this same distinct lipid kinase family (17, 18). Members of this novel lipid kinase family have several conserved regions within their primary sequences. Using a reverse transcription-PCR method involving degenerate primers corresponding to these highly conserved sequences, we succeeded in identifying a novel PIPK isoform and named it PIPKIIγ.

**Fig. 4.** PIPKIIγ is a phosphoprotein. A, Western blot analysis of lysates from rat brain, PC12 cells, 3Y1 cells, and PIPKIIγ-overexpressing COS-7 cells. The PIPKIIγ overexpressed in COS-7 cells used here was not the tagged form. B, alkaline phosphatase treatment of immunoprecipitated Myc-tagged PIPKIIγ. Immunoprecipitated Myc-PIP KIIγ was incubated with 2 units of calf intestine alkaline phosphatase (CIAP) at 30°C for 60 min. The resulting products were detected by Western blotting with anti-Myc antibody. C, 32P labeling experiment. PC12 cells were metabolically labeled with [32P]orthophosphate. PIPKIIγ was immunoprecipitated (IP) from the labeled cell lysates and detected by autoradiography or Western blotting with anti-PIPKIIγ antibody. h.c., heavy chain; IB, immunoblotting. D, phosphoamino acid analysis of PIPKIIγ. The immunoprecipitated PIPKIIγ used in B was hydrolyzed with 6 N HCl for 1 h at 110°C. The resulting amino acids were separated by TLC electrophoresis and detected by autoradiography. The positions of standard phosphoamino acids and free orthophosphate are also indicated. E, PIPK activity of Myc-PIP KIIγ after calf intestine alkaline phosphatase treatment. Immunoprecipitates treated with calf intestine alkaline phosphatase as described for B were washed with reaction buffer for PIPK, and the reaction was carried out. Results are representative of three independent experiments.

Although PIPKIIγ seems to belong to the type II subtype, the similarity between PIPKIIγ and other members of the type II PIPK family is not very high (61.1% for IIα and 63.3% for IIβ) compared with the homology between PIPKIIα and PIPKIIβ (76.7%). This, together with the difference in its expression pattern from that of other PIPKs, suggests that PIPKIIγ has some distinct functions in vivo.

PIP KIIγ was detected as a doublet migrating protein by Western blotting with a specific polyclonal antibody not only in rat brain lysates, but also in 3Y1 fibroblasts and PC12 cells. The same doublet patterns were also observed when PIPKIIγ was overexpressed in COS-7 cells. The evidence presented in this study shows that PIPKIIγ is phosphorylated in vivo and that the upper band represents the phosphorylated form. Furthermore, phosphoamino acid analysis revealed that phosphorylation occurs predominantly on serine residues. We also observed that mitogens such as serum and growth factors immediately induced phosphorylation of PIPKIIγ. The total cellular amount of PIP and the PIPK activity have been reported to increase in response to various extracellular stimuli, including EGF (5), formyl-methionyl-leucyl-phenylalanine, platelet-activating factor (1), thrombin (22), phorbol ester (4),...
and adhesion to fibronectin (6). Some of these extracellular stimuli have been reported to increase PIPK activity, especially in the cytoskeleton. In addition, the involvement of G-proteins, including small GTPases such as Rac and Rho, has also been suggested by data showing that the PIPK activity is potenti- 
ed by non-hydrolyzable GTP or is associated with recombi-
nant Rho and Rac proteins. Despite the above observations, the 
role of PIPK activity downstream of extracellular stimuli has been reported to increase PIPK activity, especially in the cytoskeleton. It may be important for this minor phosphoinositide 
to be localized in a restricted area such as in microsomes with its metabolizing enzyme, PIP5K, for efficient PI(4,5)P2 
metabolism. PIPKs have been reported to be localized in the microsomal fraction. Wong et al. 
(25) reported that PI 4-kinase α is localized in the ER, whereas PI 4-kinase β is localized in the Golgi apparatus in HeLa cells. Most PI syn- 
thase activity is also detected in the ER (24, 26, 27). It is conceivable that PI(4,5)P2 synthesis occurs efficiently in 
microsomes because of the relay of substrates between PI syn-
thesis, PI kinase, and PIPK. In addition, PIP5K, the preferential 
substrate for type II isoforms in PI(4,5)P2 synthesis, is rare in 
NIH3T3 cells (21) compared with PI4P, which exits abundantly in 
the cell. It may be important for this minor phosphoinositide 
to be localized in a restricted area such as in microsomes with its 
metabolizing enzyme, PIP5K, for efficient PI(4,5)P2 
synthesis. Many of the characteristics of PIP5K have yet to be 
elucidated, including its synthetic pathway as well as the identity 
of PI 5-kinase and its exact intracellular localization. How-
ever, together with the observation that PIPKIIγ is localized in the 
ER after phosphorylation by mitogenic signals, our results 
suggest that PIPKIIγ is involved in the synthesis of PI(4,5)P2 
in the ER.

Shibasaki et al. (28) reported that the type I PI4P 5-kinase 
overexpressed in COS-7 cells by an adenovirus expression sys-
tem is localized mainly in plasma membranes and the cytosol. They further reported that type I PIPKs induce a pine needle-
like structure of the actin cytoskeleton downstream from Rho. 
In contrast, we observed no change in the actin cytoskeleton 
when type IIβ and IIγ isoforms were transiently overexpressed 
in COS-7 cells (data not shown). From these results, it is 
possible to conclude that each subfamily of PIPK has a distinct 
localization and function and is also responsible for the syn-
thetis of distinct intracellular PIP3 sources.

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FIG. 6. Intracellular localization of PIPKIIγ. A, immunofluores-
ence of PIPKIIγ. 3Y1 fibroblasts were fixed with 3.7% formaldehyde 
and permeabilized with 0.2% Triton X-100. Then, the cells were stained 
with anti-PIP2 antibody (PIP2g), anti-BiP antibody (BiP), and 
rhodamine-conjugated wheat germ agglutinin (WGA). The overlapping 
image is also presented (merge). B, subcellular fractionation of PIPKIIγ 
in rat liver. Rat liver was subjected to subcellular fractionation as 
described under “Experimental Procedures.” About 20 μg of protein in 
each fraction (cytosolic, smooth microsomal (SM), and rough microso-
mal (RM)) was subjected to Western blotting by anti-PIP2g, anti-BiP, 
and anti-β-tubulin antibodies. C, serum-starved 3Y1 fibroblasts were 
stimulated with 100 ng/ml EGF for 10 min, and then subcellular 
fractionation was carried out. Detection of each protein was done as de-
scribed for B.
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