Type I Phosphatidylinositol-4-phosphate 5-Kinases

CLONING OF THE THIRD ISOFORM AND DELETION/SUBSTITUTION ANALYSIS OF MEMBERS OF THIS NOVEL LIPID KINASE FAMILY*

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Hisamitsu Ishihara‡‡, Yoshikazu Shibasaki‡‡, Nobuaki Kizuki‖, Takako Wada‖, Yoshio Yazaki‖, Tomoichiro Asano‡, Yoshitomo Oka‡

From the ‡Third Department of Internal Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan, and the ‖Third Department of Internal Medicine, Yamaguchi University School of Medicine, Kogushi, Ube, Yamaguchi 755, Japan

Type I phosphatidylinositol 4-phosphate (PtdIns(4)P) 5-kinases (PIP5K) catalyze the synthesis of phosphatidylinositol 4,5-bisphosphate, an essential lipid molecule in various cellular processes. Here, we report the cloning of the third member (PIP5Kγ) and the characterization of members of the type I PIP5K family. Type I PIP5Kγ has two alternative splicing forms, migrating at 87 and 90 kDa on SDS-polyacrylamide gel electrophoresis. The amino acid sequence of the central portion of this isoform shows approximately 80% identity with those of the α and β isoforms. Northern blot analysis revealed that the γ isoform is highly expressed in the brain, lung, and kidneys. Among three isoforms, the β isoform has the greatest Vmax value for the PtdIns(4)P kinase activity and the γ isoform is most markedly stimulated by phosphatidic acid. By analyzing deletion mutants of the three isoforms, the minimal kinase core sequence of these isoforms were determined as an approximately 380-amino acid region. In addition, carboxy-terminal regions of the β and γ isoforms were found to confer the greatest Vmax value and the highest phosphatidic acid sensitivity, respectively. It was also discovered that lysine 138 in the putative ATP binding motif of the α isoform is essential for the PtdIns(4)P kinase activity. As was the case with the α isoform reported previously (Shibasaki, Y., Ishihara, H., Kizuki, N., Asano, T., Oka, Y., Yazaki, Y. (1997) J. Biol. Chem. 272, 7578–7581), overexpression of either the β or the γ isoform induced an increase in short actin fibers and a decrease in actin stress fibers in COS7 cells. Surprisingly, a kinase-deficient substitution mutant also induced an abnormal actin polymerization, suggesting a role of PIP5Ks via structural interactions with other molecules.

Recent advances in cell biology have revealed that phosphoinositide metabolism plays an essential role in various cellular processes. Synthesis and breakdown of certain phosphoinositides at appropriate times and intracellular sites appear to be required for complex regulation of these cellular processes. One of the phosphoinositides, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2),1 is located at an important branchpoint in phosphoinositide metabolism. PtdIns(4,5)P2 serves as a substrate for phosphoinositide-specific phospholipase C (EC 3.1.4.11), generating the second messengers 1,2-diacylglycerol and inositol 1,4,5-trisphosphate (1). PtdIns(4,5)P2 can also be phosphorylated by phosphoinositide 3-kinase (EC 2.7.1.137), generating phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3). The synthesis of which is activated in signaling pathways of several growth factor receptors (2). Furthermore, PtdIns(4,5)P2 modulates the activity of numerous enzymes, including actin-binding proteins (3), binds pleckstrin homology domains (4–6), and has been suggested to play a role in exocytosis (7–9). The major pathway of PtdIns(4,5)P2 synthesis is that mediated by type I phosphatidylinositol 4-phosphate 5-kinases (PIP5K; EC 2.7.1.68), phosphorylating the D-5 position of the inositol ring of phosphatidylinositol 4-phosphate (PtdIns(4)P). Despite these important functions of PtdIns(4,5)P2, direct investigations of the intracellular roles and of mechanisms regulating synthesis of this lipid molecule are limited. Until recently, a major factor hindering progress in this field was the absence of molecular tools.

Recently, cDNAs encoding two isoforms of type I PIP5K have been cloned (10, 11). Herein, we report molecular cloning of a third isoform of type I PIP5K (PIP5Kγ) from a cDNA library of the murine pancreatic β-cell line MIN6 (12). This novel isoform has two alternative splicing forms of 87 and 90 kDa and is the most markedly stimulated by phosphatidic acid of the three isoforms. These molecular identifications revealed that PIP5K isoforms constitute a novel lipid kinase family, distinct from phosphoinositide 3-kinases, phosphatidylinositol 4-kinases, and diacylglycerol kinases. Demonstration of structural characteristics is essential for understanding the intracellular roles of these isoforms and the mechanisms by which they are regulated. Therefore, in this report, several aspects of the structural characteristics of these isoforms were also studied in vitro and in vivo. We found that a central region, consisting of approximately 380 amino acids, is sufficient for PtdIns(4)P kinase activity and that carboxyl-terminal regions are important for modulation of the kinase activities of these isoforms. We also found that expression of either the β or the γ isoform leads to actin rearrangement in COS7 cells, as was the case.

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ, EBI, and GenBankTM Data Bank with accession number(s) AB009616 and AB009615.

† To whom correspondence should be addressed: Third Department of Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Tel.: 81-3-3815-5411 (ext. 3121); Fax: 81-3-5803-1874; E-mail: isihihara-tky@umin.u-tokyo.ac.jp.

‡ The abbreviations used are: PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(5)P, phosphatidylinositol 5-phosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PIP5K, phosphatidylinositol-4-phosphate 5-kinase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; HA, hemagglutinin; PKA, cyclic AMP-dependent protein kinase.
with the α isoform (13), and that the central region is sufficient for this effect. Furthermore, surprisingly, the expression of a kinase-deficient substitution mutant generated a similar effect in COS7 cells.

**EXPERIMENTAL PROCEDURES**

Cloning of Murine Type I PIP5K—A polymerase chain reaction (PCR) using degenerate oligonucleotide primers and screening of a MIN6 cell cDNA library were as described previously (10). To obtain a 5′ sequence of PIP5Kγ, a Marathon™ cDNA amplification kit (CLONTECH) was used according to the instructions of the manufacturer. The reverse transcription was performed using M-MuLV-MIN6 cell poly(A) RNA and an antisense primer 5′-GTTGACGTAGAAGACAGAGC-3′. The first PCR was performed using adapter primer 1 (CLONTECH) and an antisense primer, 5′-CTTCACTGGGAAAGAGAGA-3′. The second PCR was performed using adapter primer 2 (CLONTECH) and an antisense primer, 5′-GTCGCCAGCTTCTCCGAGAG-3′. The first and second PCR reactions were conducted with inclusion of dimethyl sulfoxide (5%), without which only shorter products were obtained. Individual clones were sequenced following subcloning into pGEM-T vector (Promega, WI) as described above. The consensus of three independent clones confirms the sequence of the 5′ region of PIP5Kγ.

**Production of Antibody Specific to the γ Isoform and Western Blotting**—A murine multiple tissue Northern blot (CLONTECH) was hybridized according to the instructions of the manufacturer with an [α-32P]dCTP-labeled 0.4-kb Aor51I-HiPatt fragment from the 3′ portion of the γ isoform cDNA.

**Isoform and Western Blotting**—An oligopeptide, CASDEEDAPSTDIYF, was custom synthesized (15–17). COS7 cells (1.5 × 106 cells) maintained in Dulbecco’s modified Eagle’s medium with 10% FBS, 5% calf serum, 10% 15M ammonium hydroxide and incubated with anti-HA monoclonal antibody (12CA5) in phosphate-buffered saline with 0.2% gelatin at room temperature for 45 min. After washing three times with phosphate-buffered saline-gelatin, cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (DAKO) and rhodamine-conjugated phalloidin (Molecular Probes) for 30 min. Slides were observed under a Bio-Rad confocal microscope system (MRC 1024).

**Materials**—PtdIns(4)P from bovine brain and phosphatidic acid were purchased from Sigma. Oligonucleotides were custom synthesized and purchased from either Japan Bio-service Inc. (Saitama, Japan) or Becks Inc. (Itabashi, Tokyo).

**RESULTS**

Cloning of the Third Member of Type I PIP5K Family—A polymerase chain reaction (PCR) using degenerated primers and subsequent screening of a MIN6 cell cDNA library, as described previously (10), identified a class of partial coding sequences with homology to type I PIP5K α and β isoforms. The third cDNA sequence, designated PIP5Kγ, contains four overlapping clones, one of which has an additional 78-base pair sequence at the 3′ terminus and possibly arises by alternative splicing of the PIP5Kγ gene. In-frame stop codons upstream from the first ATG codon of this cDNA could not be identified in the initial study. Therefore, to obtain an additional 5′ sequence, an adapter ligation/PCR-based method (Marathon™, CLONTECH) was employed. Although an additional 172 base pairs and another ATG codon were obtained, there were no in-frame stop codons in a 79-base pair sequence upstream from this ATG codon. Nonetheless, this ATG codon was concluded to be the initial translation codon for the following reasons. First, the ATG codon is in a favorable position for translation according to Kozak’s rules (Fig. 1, Ref. 19). Second, as shown in Fig. 2A, recombinant proteins of the γ isoform with or without the 26 carboxy-terminal amino acids expressed via adenoviral vectors migrated almost identically to either of the doublet bands (87 and 90 kDa) from brain tissue on SDS-PAGE. The γ isoform has two alternative splicing forms, consisting of 635 and 661 amino acids with calculated molecular masses of 69,563 and 72,469 Da, and isoelectric points of 5.40 and 5.27, respectively (Fig. 1). Because the 87-kDa protein was predominantly expressed in brain tissue (Fig. 2A) and MIN6 cells (data not shown), the γ isoform without the 26 carboxy-terminal amino acids was used in subsequent analyses. As shown in Fig. 3, the central portions of the three type I isoforms were found to be very similar (approximately 80% identity) in amino acid sequence. In addition, the carboxy-terminal 39 amino acids of the γ isoform shows partial homology with that of the β isoform (approximately 40% identity) whereas the carboxy-terminal regions differed in length and amino acid sequence among the three isoforms. An entire coding sequence of murine cDNA homologous to human PIP5KIId (20, 21), which was recently revealed to be phosphatidylinositol 5-phosphatase (PtdIns(5)P)
4-kinase (22), was also cloned from a MIN6 cell cDNA library (data not shown). The murine PIP5KIIα consists of 405 amino acids, one residue less than its human counterpart, with only seven conserved amino acids differing between the two (Fig. 3).

Tissue Distribution of Type I PIP5K—Northern blotting analysis was performed using the γ isoform cDNA probe corresponding to the sequence close to the termination codon. A 4.8-kilobase mRNA was detected, as a major band, in murine poly(A) RNA from different tissues (Fig. 2B). The tissue distribution of the γ isoform differed from those of the α and β isoforms, being essentially restricted to the brain, lung, and kidneys.

The Novel cDNA Encodes Type I PIP5K Protein—To characterize the enzymatic activity of the third isoform, HA-tagged proteins of this isoform expressed in COS7 cells were immunoprecipitated using anti-HA-epitope monoclonal antibody 12CA5. The resulting immunocomplex exhibited PtdIns(4)P kinase activity (Fig. 2C). Although this thin layer chromatography separation did not provide information about whether the PtdIns(4)P produced was PtdIns(4,5)P2 or PtdIns(3,4)P2, the close sequence similarity with the α and β isoforms (Fig. 3) strongly suggests that the γ isoform is also a 5-kinase. Furthermore, the PtdIns(4)P kinase activities of the third isoform increased by more than 10-fold when an equimolar amount of phosphatidic acid was added to the reaction solutions (Fig. 2D), demonstrating the novel murine cDNA to encode the type I PtdIns(4)P 5-kinase (23, 24).

Comparison of Kinetic Activities of Type I PIP5K Isoforms—For initial characterization of members of the PIP5K family, kinetic parameters for the PtdIns(4)P kinase activity of these murine isoforms were studied. For this purpose, recombinant proteins of isoforms with the HA epitope were expressed and immunoprecipitated with the anti-HA-epitope monoclonal antibody. One-half of each immunoprecipitate was used for kinase assay and the other half for Western blotting with rabbit anti-HA-epitope polyclonal IgG. Lipid kinase activity was normalized with the protein amount estimated by Western blotting (for example, see Fig. 5B). Kinetic parameters for these isoforms are summarized in Table I. While affinities for PtdIns(4)P and ATP were similar among the three isoforms, the β isoform had the greatest Vmax value, approximately 3.2-fold and 1.7-fold higher than those of the α and γ isoforms, respectively.
and a 46-amino acid deletion virtually abolished kinase activity. 

The data suggest that an approximately 380 amino acid central portion of the PIP5Kα isoform (amino acid residues 18 to 399) constitutes the kinase core domain. Indeed, as described below, this central portion alone retains kinase activity (see Fig. 6B). This region of the PIP5Kα isoform has about 80% amino acid identity with the corresponding regions of both the β and the γ isoform. The amino-terminal half of the kinase core domain is especially conserved among the three isoforms (more than 90%) (Fig. 3). In addition, sequence alignment between type I PIP5Ks and PIP5KIIAs suggests that the latter enzyme (PtdIns(5)P 4-kinase) consists essentially of the kinase region of type I PIP5Ks with approximately 40% identity (Fig. 3).

Mutations in the Putative Nucleotide Binding Region—In several protein and lipid kinases, a glycine-rich sequence followed by a lysine residue 10–30 residues downstream constitutes an important region for phosphate-transfer reactions (25, 27). Although there is no typical region for such a glycine-rich sequence, the region of amino acid residues 121 to 138 in the α isoform is similar to the ATP binding domain of cyclic AMP-dependent protein kinase (PKA). To examine the role of this region, two α isoform mutants were constructed in which glycine 124 was substituted with valine (PIP5Kα-G124V mutant) or lysine 138 with alanine (PIP5Kα-M138A mutant). A lipid kinase assay revealed type I PIP5Kα-G124V to have 67 ± 11% of the PtdIns(4)P kinase activity of the wild-type protein, while type I PIP5Kα-K138A had virtually no kinase activity (Fig. 5B).

Role of Amino- and Carboxyl-terminal Variable Regions of Type I PIP5Ks—As indicated above, these type I PIP5K isoforms consist of a similar central domain and variable amino- and carboxyl-terminal regions. To characterize these domains, amino- and/or carboxyl-terminal deletion mutants of the three isoforms, carrying the HA-epitope, were constructed based on the results of deletion analysis of the α isoform (Fig. 6A). As summarized in Fig. 6B, the central regions of the three isoforms showed essentially equivalent lipid kinase activities. There were no marked changes when amino- and/or carboxyl-terminal regions were deleted from the α isoform. As shown in Table I, the β isoform had approximately three-fold higher activity than the α isoform. When the carboxyl-terminal region was deleted from the β isoform, the activity was reduced to a level approaching that of the α isoform. In addition, a deletion of the carboxyl-terminal region of the γ isoform also resulted in a reduction in its kinase activity. The phosphatidic acid sensitivities of these deletion mutants were also examined (Fig. 6C). The central regions alone of the three isoforms can be stimulated by phosphatidic acid. Although the γ isoform showed the highest sensitivity to phosphatidic acid, its carboxyl-terminal deletion mutant exhibited a magnitude of phosphatidic acid stimulation similar to that of the α isoform.

Effects on Actin Polymerization of Overexpressing PIP5K Isoforms and Their Mutants in COS7 Cells—As reported previously, overexpression of type I PIP5Kα via an adenoviral vector led to a decrease in typical stress fibers and an increase in disassembled short actin fibers (13). In this study, we examined the effects of overexpressing three type I PIP5K isoforms, type II PIP5Kα, and their mutants on actin polymerization (Fig. 7). Abnormal reorganization of actin fibers was also observed in COS7 cells overexpressing either the β or the γ isoform (Fig. 7, B and C). These in vivo analyses using deletion mutants of the α isoform are summarized in Fig. 4. All deletion mutants of the α isoform with the complete kinase core domain induced abnormal actin reorganization in COS7 cells. In addition, COS7 cells expressing Iα-Ka, Iβ-Ka, or Iγ-Ka mutants in which both amino- and carboxyl-terminals are deleted from the three isoforms (Fig. 6A), had enormous amounts of short actin

respectively. Study of phosphatidic acid sensitivity revealed the γ isoform to be most sensitive to phosphatidic acid.

Deletion Analysis of the Type I PIP5Kα Defines a Central Kinase Domain—Because type I PIP5K isoforms have no sequence homology with other lipid kinases including phosphoinositide 3-kinases, phosphatidylinositol 4-kinases and diacylglycerol kinases (25, 26), it is of great importance to determine their structure and function relationships. To begin to address this issue, amino- or carboxyl-terminal deletion mutants of the type I PIP5Kα isoform were constructed using endogenous restriction enzyme sites and PCR-based methods. As summarized in Fig. 4, while all four mutants with stepwise deletions from the carboxyl terminus to glutamine residue 400 have activity almost equal to that of the wild-type α isoform, the PIP5Kα-(1–392)/EcoRI mutant has little or no PtdIns(4)P kinase activity. In contrast to the long dispensable region in the carboxyl terminus, amino-terminal deletions had a pronounced effect. Although the first 17-amino acid deletion did not alter kinase activity, deletion of only 31 amino acids from the amino-terminal region resulted in significantly reduced kinase activity (34 ± 9% of the full-length α isoform, mean ± S.E., n = 3), and a 46-amino acid deletion virtually abolished kinase activity.

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fibers and relatively few stress fibers (data not shown). In contrast, PIP5Kα-(1–392)/EcoRI and PIP5Kα-(47–539), mutants with small deletions at the carboxyl- and amino-termini, respectively, of the kinase core domain failed to induce abnormal actin rearrangement (Fig. 7E). All other deletion mutants with the incomplete kinase core domain (Fig. 4) and type II PIP5Kα (data not shown) exhibited behaviors similar to those of PIP5Kα-(1–392)/EcoRI and PIP5Kα-(47–539).

These data appeared to indicate that kinase activity of PIP5K would be necessary for abnormal actin polymerization. However, surprisingly, expression of a kinase-deficient substitution mutant, PIP5Kα-K138A, also led to a decrease in typical stress fibers and an increase in short actin fibers (Fig. 7F). In addition, as was the case with the wild-type α isoform (13), COS7 cells expressing the kinase defective substitution mutant also exhibited decreased adhesion activity. They became rounded and readily detached from the bottoms of culture dishes (data not shown).

| TABLE I |
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| Kinetics of type I PIP5K isoforms |
| The relative Vmax for PtdIns(4)P kinase were obtained after normalization of the Vmax values with protein amounts estimated by Western blotting. The normalized Vmax value of the α isoform was taken as 1.0. Kα for PtdIns(4)P was measured at a ATP concentration of 25 μM and varying concentrations of PtdIns(4)P. Kμ for ATP was measured at a PtdIns(4)P concentration of 100 μM and varying concentrations of ATP. Phosphatidic acid (PA) sensitivity was measured in the presence of 100 μM of PA. Data are presented as means ± S.E. of five independent experiments each performed in triplicate. |
| **Relative Vmax for PtdIns(4)P** | **Kα for PtdIns(4)P** | **Kμ for ATP** | **PA sensitivity (fold stimulation)** |
|---|---|---|---|
| PIP5Kα | 1.0 | 34 ± 7 | 27 ± 7 | 9.2 ± 1.0 |
| PIP5Kβ | 3.2 ± 0.7 | 26 ± 8 | 33 ± 8 | 8.4 ± 1.5 |
| PIP5Kγ | 1.9 ± 0.4 | 37 ± 7 | 39 ± 8 | 13.7 ± 1.4 |
DISCUSSION

A novel isoform of PIP5K was identified in this study. This novel isoform (PIP5Kα) migrated at 90-87 kDa on SD-PAGE, suggesting this isoform to be identical or closely related to the type Ib isoform (90 kDa) previously purified (23). The coexistence of three isoforms of the type I PIP5K in insulin-secreting clonal cells suggests that these isoforms have specific functions in vivo. Since the 90-kDa isoform has been reported to have higher activity than the 68-kDa isoform in restoring Ca^2+ sensitivity to phosphatidic acid of the PIP5K (8), the highest activity is most markedly stimulated by phosphatidic acid. Our results showed that the 90-kDa isoform has the highest activity when 10–70%, and the 68-kDa isoform when more than 70% cells expressing one of deletion mutants exhibited an increase in short actin fibers and a decrease in stress fibers, as when 10–70%, and when less than 10% (for typical cells, see Fig. 7).

We also found that overexpression of any one of the three isoforms led to the production of massive amounts of short actin fibers while disrupting actin stress fibers in COS7 cells. A surprising result was that a kinase deficient mutant, PIP5Kα-K138A, induces similar effects. The mechanism by which PIP5K isoforms and the kinase-deficient substitution mutant induce such effects remains to be determined. The causal relationship between short actin fiber formation and disruption of actin stress fibers is also unclear. It has been reported that expression of PtdIns(4,5)P_2 5-phosphatases in COS7 cells decreased the number of actin stress fibers via the hydrolysis of PtdIns(4,5)P_2 bound to actin regulatory proteins (13, 30). An
opposite mechanism (i.e. via an increase in PtdIns(4,5)P$_2$) is unlikely to lead to an increase in short actin fibers in cells overexpressing 5-kinases since the kinase-deficient mutant induced a similar effect, although the possibility of endogenous 5-kinase activity playing some part in the effect cannot be ruled out. Indeed, it was reported that overexpression of type I PIP5K isoforms in COS7 cells did not increase cellular levels of PtdInsP$_2$ (31), providing evidence that effects on actin reorganization of overexpressing PIP5Ks were not mediated by the kinase activity of overexpressed proteins. The fact that the kinase inactive mutant induces actin reorganization similar to that seen with the wild-type enzyme suggests that structural interactions with other as yet unknown molecules mediate this effect. Small GTP binding proteins, Rac and Rho, are possible candidates (32–34). In this regard, it should be noted that the structure of the kinase core domain was found to be sufficient
for inducing abnormal actin polymerization. There may be a binding site for such an interacting molecule within the kinase core domain. Future studies should be designed to identify the molecules interacting with PIP5K isoforms.

Recent findings suggest that PIP5Ks play various roles in signaling pathways, by participating in the synthesis of a number of phosphoinositides (28). The present results suggest that structural interactions are also important in PIP5Ks functions. Much research remains to be done in order to elucidate the complex signaling pathways in which these lipid kinases are involved.

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