Type I Phosphatidylinositol-4-phosphate 5-Kinases Are Distinct Members of This Novel Lipid Kinase Family*

Recombinant, bacterially expressed PIP5KI bp, base pair(s); IPTG, isopropyl-β-D-thiogalactopyranoside; RACE, rapid amplification of cDNA ends; kb, kilobase(s) pair; phatidylinositol 4-phosphate; HPLC, high performance liquid chromatography; RACE, rapid amplification of cDNA ends.

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Phosphatidylinositol-4-phosphate 5-kinases (PIP5K) synthesize phosphatidylinositol-4,5-bisphosphate, a key precursor in phosphoinositide signaling that also regulates some proteins and cellular processes directly. Two distinct PIP5Ks have been characterized in erythrocytes, the 68-kDa type I (PIP5KI) and 53-kDa type II (PIP5KII) isoforms. Using peptide sequences from the erythroid 68-kDa PIP5KI, we have isolated cDNAs encoding PIP5KI in normal human brain. Partial cDNAs obtained for a second isoform, PIP5KIIα, established that the human STM7 gene encoded a previously unrecognized PIP5KI. However, the peptide sequences demonstrated that erythroid PIP5KI corresponded to PIP5KIα. Recombinant, bacterially expressed PIP5KIα possessed PIP5K activity and was immunoreactive with erythroid PIP5K antibodies. By Northern analysis, PIP5Kα and PIP5KIIα had wide tissue distributions, but their expression levels differed greatly. PIP5Ks had homology to the kinase domains of PIP5KIIα, yeast Mss4p and Fab1p, and a new Caenorhabditis elegans Fab1-like protein identified in the data base. These new isoforms have refined the sequence requirements for PIP5K activity and, potentially, regulation of these enzymes. Furthermore, the limited homology between PIP5Ks and PIP5KIIα, which was almost exclusively within the kinase domain core, provided a molecular basis for distinction between type I and II PIP5Ks.

Phosphatidylinositol-4-phosphate 5-kinases (PIP5K) synthesize phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) by phosphorylating phosphatidylinositol 4-phosphate (PtdIns4P). This conversion is pivotal to the phosphoinositide cycle as PtdIns(4,5)P2 is hydrolyzed by phosphoinositide-specific phospholipase C to generate the second messengers 1,2-diacylglycerol and inositol 1,4,5-trisphosphate. These second messengers activate several protein kinase C isoforms and effect the release of stored intracellular calcium, respectively. PtdIns(4,5)P2 is also phosphorylated by agonist-activated phosphatidylinositol 3-kinases, resulting in synthesis of phosphatidylinositol 3,4,5-trisphosphate, a second messenger whose targets are largely unknown, but may include protein kinase C isoforms (1–3). In addition, PtdIns(4,5)P2 directly modulates the function of proteins, including phospholipase D (4) and many actin-binding proteins (5). It binds pleckstrin homology domains found in some signaling proteins (6, 7) and is required for Ca2⁺-regulated secretion of neurotransmitters (8). The diversity of PtdIns(4,5)P2 actions suggests that PIP5Ks are crucial enzymes for many cellular functions (reviewed in Ref. 9).

Multiple PIP5K isoforms have been purified and biochemically characterized (9–13). The best characterized isoforms are the erythroid enzymes which have been categorized as type I or type II PIP5Ks based on their separation by phosphocellulose ion exchange (10–12). These PIP5Ks differ in molecular mass and are biochemically and immunologically distinct. Two notable differences are that PIP5KI, but not PIP5KII, can be stimulated by phosphatidic acid and can use PtdIns4P present in membranes as a substrate (10, 11, 13). PIP5KII and PIP5KIIα also seem to be functionally distinct.

PIP5KI, but not PIP5KII, isoforms have been implicated in the secretion of neurotransmitters and small G-protein regulation. Neurotransmitter release from permeabilized PC12 cells has been divided into an initial Mg⁺-ATP-dependent step, followed by a Ca2⁺*-triggered exocytotic event (8, 14, 15). PIP5KI isoforms, and the production of PtdIns(4,5)P₂, are required for the priming step, while PIP5KII is ineffective (8). PIP5KI isoforms also appear to associate with, and are putatively stimulated by, the small G-proteins Rac and Rho (16–18). As above, type II PIP5Ks do not interact with these G-proteins (16–18). Besides being important regulators of cytoskeletal assembly, Rac and Rho have been linked to secretion and certain growth factor receptor signaling cascades, all processes known to involve PIP5Ks and PtdIns(4,5)P₂ (19–24). The direct action of PtdIns(4,5)P₂ and PIP5Ks in these diverse processes argues that the kinases are tightly regulated components of these signaling pathways and not merely passive replenishers of phosphoinositide pools.

The recent isolation of a cDNA encoding a human type II phosphatidylinositol-4-phosphate 5-kinase (PIP5KIIα) established a novel lipid kinase family with two yeast homologues, Mss4p and Fab1p (25). We have now expanded this family by isolating the first type I PIP5K cDNAs and identifying a Fab1p homologue in Caenorhabditis elegans. The cDNAs and sequences of these crucial enzymes will permit studies into the unique biochemical properties of the PIP5KII subfamily and how they integrate into signaling pathways, cytoskeletal control, and secretion.
**EXPERIMENTAL PROCEDURES**

**Purification of PIP5KI and Peptide Sequencing—** PIP5KI was purified from bovine erythrocytes by phosphocellulose chromatography as described previously (11). The 68-kDa PIP5KI was cleaved with CNBr, separated by SDS-polyacrylamide gel electrophoresis, and transferred to Immobilon membranes (Millipore). Peptides were sequenced directly from these membranes by N-terminal Edman degradation at the University of Wisconsin Biotechnology Center. A PIP5KI purification procedure of 53 kDa was also purified by phosphocellulose chromatography. This 53-kDa protein was electroeluted from gel slices after SDS-polyacrylamide gel electrophoresis and digested with lysyl endopeptidase (Wako). These peptides were purified by HPLC on a reverse phase Aquapore RP-300 (ABI) column using an acetonitrile gradient in the presence of 0.1% trifluoroacetic acid. The eluted peptide peaks were collected and sequenced by Edman degradation as outlined previously.

**Cloning of PIP5KI cDNAs—** The PIP5KI peptide sequences were searched against the sequence data bases using the BLAST algorithm (26). One peptide of the bovine 68-kDa PIP5KI matched an expressed sequence tag from human infant brain (HFBEM40, accession number T07661) which was ordered from the American Type Culture Collection. This partial cDNA was used as a probe for library screening performed by standard techniques (27). After isolating partial cDNAs with additional 5’ sequences from a human placenta λgt11 library, primers were constructed for 5’ RACE using a human placenta 5’-RACE Ready cDNA (Clontech). One of the human brain 5’-RACE products was used to screen a human fetal brain ADR2 library (Clontech) yielding six independent PIP5KI cDNAs. Expressed sequence tags for PIP5KIβ (accession numbers Z45334, F13210, and R18676) were discovered doing BLAST searches with PIP5KI nucleotide and protein sequences. Clones c-2mh06 (Z45334) and c-3kc04 (F13210) were obtained from the Genexpress cDNA program, Laboratoire Genethon, Evry, France. Clone 30430 (R18676) was acquired from the IMAGE consortium, Lawrence Livermore National Laboratory. Upon receipt, these clones were sequenced (Sequenase v2.0, U. S. Biochemical Corp.).

**Northern Blotting—** The same human Multiple Tissue Northern blot (Clontech) was probed for PIP5KI, PIP5KIβ, and β-actin following the manufacturer’s instructions. The DNA fragments used as probes corresponded to base pairs 505-1043 or 1425-2019 of PIP5KIα, or 589-1081 of PIP5KIβ clone c-2mh06 (1339–1786 of STM7). The fragments were gel-purified after restriction enzyme digestion and labeled with [α-32P]ATP by random priming with Klenow DNA polymerase (27).

**Expression of PIP5KI in Escherichia coli—** PIP5KIα1 was subcloned into the pET28b expression vector (Novagen) as follows. The 1.6-kb open reading frame of PIP5KIα1 was amplified by PCR using Tak polymerase and the primers CTTGGATCCTAAGGCGTCGGCC (forward) and GGGAATTCCGTATTTAGGGTGACCTCTG (reverse). The forward primer annealed over the initiation codon and included a BamHI site, while the reverse primer overlapped the termination codon and introduced an EcoRI site. The PCR products were subcloned into pT7Blue (Novagen) and screened by colony hybridization with a 32P-labeled DNA fragment from PIP5KIα1, or 589-1081 of PIP5KIβ clone c-2mh06. The entire cDNA encoding the B1-isoform was subcloned into pET28b (pET-PIPKIα1). The hexahistidine-tagged fusion protein created is identified as His-PIPKIα1.

The E. coli strain BL21 (DE3) was transformed with either pET-PIPKIα1 or pET28b and expression could be induced by IPTG. Expression procedures essentially followed those recommended by the manufacturer, 6th Ed. (Novagen). Samples were prepared for Western blotting from both induced and uninduced cultures of these cells. For activity assays, extracts of these cultures were separated into 0.1% Triton X-100-soluble and -insoluble fractions. The majority of the expressed His-PIPKIα1 was present in the insoluble pellet fraction, which includes inclusion bodies. On a larger scale, His-PIPKIα1 was purified by Ni2+-chelate chromatography using His-Bind resin (Novagen). The eluted His-PIPKIα1 was dialyzed against buffer containing 50 mM Tris, pH 7.5, 1 mM EGTA, and 10% glycerol.

**Kinase Activity Assays and Western Blotting—** The soluble fractions of induced and uninduced cells, or partially purified His-PIPKIα1, were assayed for PIP5K activity as described previously (11). Kinase activity was measured after completion of the reactions performed for 5.5 min at room temperature in a final concentration of 50 mM Tris, pH 7.5, 1 mM EGTA, 10 mM MgCl2, 50 µM PtdIns4P (Sigma), 50 µM ATP, and 10 µCi of [γ-32P]ATP. The labeled products, separated by thin layer chromatography, were detected by autoradiography. When testing for phosphatidic acid stimulation, 80 µM phosphatidic acid (Sigma) in the presence of 0.1% Triton TX-100 was preincubated with the kinase for 10 min at room temperature before performing the kinase assay (11).

We then performed the following established procedures after transfer to polyvinylidene difluoride membranes (28). Either a polyclonal PIP5KI antibody (10 µg/ml) or the T7 Tag monoclonal antibody (1:10,000) was used to detect His-PIPKIα1. The PIP5KI antibody had been raised against the bovine 68-kDa isoform and purified by affinity chromatography using that antigen (11). The primary antibody was detected by chemiluminescence using a horseradish peroxidase-conjugated secondary antibody (Santa Cruz) and Lumiglo substrates (Kirkegaard & Perry Laboratories).

**RESULTS AND DISCUSSION**

**Isolation of cDNAs Encoding PIP5KI—** An amino acid sequence obtained from the bovine erythrocyte 68-kDa PIP5KI was used to identify an expressed sequence tag in the data bases. Screening of a human fetal brain cDNA library yielded six independent PIP5KIα1 clones. Four of these clones appeared to be full-length and were extensively analyzed. The 3731 bp PIP5KIα1 clone, encoded by two cDNAs, had a 549-amino acid residue open reading frame, shown in Fig. 1. The calculated molecular mass of the encoded protein was 61,186 Da. The putative start site was in the context of a good Kozak sequence (29) and was preceded by four in-frame stop codons. Two other cDNA clones appeared to be the same as PIP5KIα1 except that PIP5KIα2 had a 36-bp insertion, while PIP5KIα3 had a deletion of 147 bp. These transcripts likely arise by alternative splicing of the PIP5KI gene. If this proves to be true, these changes would result in the addition of 12 amino acids near the N terminus of PIP5KIα2 and removal of 49 amino acids from the C terminus of PIP5KIα3 and the conversion of a codon to a cysteine. Their predicted molecular masses would be 62,561 Da and 56,052 Da, respectively.

Based on the sequence of peptides from bovine erythrocyte 68-kDa PIP5KI, it was clear that this cDNA encoded a type I PIP5K. As shown in Fig. 1, these peptides were distributed throughout the coding region and all matched with >90% identity. Ambiguous residues and potential species differences between the human and bovine proteins precluded a perfect match. Because peptide 1 (residues 34–42) spanned the PIP5KIα2-specific insertion but did not include the 12 residue insert, and peptide 13 (residues 433–443) ended within the sequences deleted from PIP5KIα3, the isoform isolated from erythrocytes must have the PIP5KIα1 sequence. Whether these potential variant PIP5KI isoforms were present in erythrocytes could not be definitively ascertained.

When the 68-kDa PIP5KI is purified from erythrocytes, a 53-kDa degradation product is often seen that is both active and immunoreactive (11). Many of the peptides shown in Fig. 1 were derived from this 53-kDa protein. As peptides from the 53- and 68-kDa proteins matched the same cDNA and actually overlapped in a few instances (peptides 5–6 and 9–11), it confirmed that they were the same protein. Indeed, as the 53-kDa protein’s peptides were distributed toward the N terminus, it suggested that removal of the C terminus of the 53-kDa protein’s peptides were distributed toward the N terminus of PIP5KIα1 and the conversion of a codon to a cysteine. Their predicted molecular masses would be 62,561 Da and 56,052 Da, respectively.

Based on the amino acid sequence, PIP5KIα1 clearly belonged in the PIP5K family of enzymes (25). As shown in Fig. 2, this type I kinase had the conserved kinase homology domain, which was 35% identical to PIP5KIα1. The concentration of invariant residues within the kinase homology domain of the PIP5K family of enzymes (110) is consistent with this region being catalytically critical as discussed below. The possible splicing variants of PIP5KIα1 were examined outside the kinase homology domain (Figs. 1 and 2A). A notable difference between PIP5KIα1 and other family members was its considerably longer C terminus. Outside of the kinase homology domain, PIP5KIα1 had
no obvious homology to other protein domains currently in the data bases.

The Isolation of PIP5KIb Identifies the Product of the STM7 Gene as a Type I PIP5K

Data base searching with PIP5Kia nucleotide and protein sequences revealed three expressed sequence tags for a second PIP5K, which we have denoted PIP5Kib. Sequence analysis of these obtained partial cDNAs indicated that they had about 70% amino acid identity with PIP5Kia. Subsequently, STM7 was isolated as a candidate gene for the human recessive disorder Friedreich's ataxia (30), now attributed to the neighboring X25 gene (31). The sequence of PIP5Kib1 was identical to that of STM7 except for a few nucleotide changes in the 3'-untranslated region (data not shown). This clearly identified the STM7 gene product as a type I PIP5K. We therefore propose that the product of the STM7 gene be called PIP5Kib.

PIP5Kib1 had a predicted molecular mass of 61,035 Da, virtually the same as PIP5Kia. These isoforms had 83% amino acid identity within the kinase domain (Fig. 2C) and 64% overall. The erythroid PIP5K peptide sequences clearly suggested that PIP5Kia, and not PIP5Kib, was isolated from erythrocytes (Fig. 1). The majority of peptides matched PIP5Kia exclusively or better than PIP5Kib, although a few peptides from the most conserved regions matched both isoforms (e.g., peptide 5). Based on Western blotting results, it seems unlikely that erythrocytes contain PIP5Kib, but this has yet to be tested definitively.

PIP5Kia and PIP5Kib were only 35% identical with PIP5KIIa within the kinase homology domain (Fig. 2C). Type I and type II PIP5Ks have little similarity outside the kinase homology domain or in the insert region. This level of conservation explains why the PIP5KI and PIP5KII antibodies do not recognize isoforms of the opposite type (11). The two PIP5KI isoforms had 83% identity, including some long matching stretches, predicting that PIP5KI antibodies would cross-react with both type I isoforms, which was indeed the case.2 Of the three PIP5Kib partial cDNAs sequenced, one (clone 30430) had a deletion of 118 bp, resulting in removal of the last 38 amino acid residues from the open reading frame (Fig. 1). This open reading frame, PIP5Kib2, would encode a predicted protein of 56,950 Da. Based on the genomic sequence of STM7 (30), PIP5Kib2 lacks exon 15 and seemed to be the same as their IIB reverse transcriptase-PCR product. It has been possible to amplify both PIP5Kib1 and PIP5Kib2 by PCR from human heart cDNA.3

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Type I Phosphatidylinositol-4-phosphate 5-Kinases

A

**FIG. 2**

**Type I Phosphatidylinositol-4-phosphate 5-Kinases**

### A

#### PIP5K1α1

- Kinase homology domain (KH)
- Inset: KH

#### PIP5K1β1

#### PIP5K1α2

#### Mss4p

#### Fab1p

#### C05E7.5

**Fab1-like protein**

### B

#### PIP5K1α1

#### PIP5K1β1

#### PIP5K1α2

#### Mss4p

#### Fab1p

#### C05E7.5

### C

| PIP5K1α | PIP5K1β | PIP5K1α2 | Mss4p | Fab1p | C05E7.5 |
|---------|---------|----------|-------|-------|---------|
| 100     | 83      | 100      | 35    | 44    | 40      |
| 35      | 34      | 100      | 44    | 40    | 43      |
| 44      | 40      | 43       | 100   | 29    | 28      |
| 29      | 27      | 28       |       |       | 28      |
| 28      | 29      | 25       |       |       | 24      |
| 24      | 28      | 28       |       |       | 28      |

### D

- **Fab1p** (S. cerevisiae)
- **C05E7.5** (C. elegans)
- **PIP5K1α**
- **PIP5K1β**
- **PIP5K1α2**
- **Mss4p** (S. cerevisiae)
terminus with 40% identity to Fab1p and also lacked the insert region (Fig. 2). Although the Fab1-like protein had some homology to Fab1p outside the kinase domain, including similarity to chaperonin TCP1 proteins (residues 196–268), it did not possess the motif identified in Fab1p that was suggested to be involved in vacuole function (33). Interestingly, BLAST data base searches of the complete Saccharomyces cerevisiae genome did not reveal any other PIP5K homologues besides Mss4p and Fab1p. This implies that these two proteins must account for all PIP5K activity and functions in this yeast.

The cloning of PIP5KI isoforms and the identification of a C. elegans homologue has increased this novel lipid kinase family to six members and refined the kinase homology domain consensus between them (Fig. 2B). Excluding the insert region, the kinase homology domain consists of approximately 300 residues, similar in size to the conserved catalytic domain of protein kinases. Of the aligned 367 residues, 33 were invariant and likely involved in catalysis or the structure of the ATP and PtdIns4P binding surfaces within the active site. It is assumed that all family members have PIP5K activity, although this has only been tested directly for PIP5KIa and PIP5KIβ (see below) and PIP5KIa (25) and indirectly for Fab1p (33). Based on similarity to nucleotide-binding proteins and protein kinases, the region around the conserved SGS sequence (residues 72–74) and Lys67 are part of a predicted nucleotide binding motif (33). The DLK sequence (residues 164–166) and Asp230 may be functionally analogous to the catalytic loop and magnesium coordination residues of protein kinases (33). The preponderance of aromatic and positively charged residues between 25–55, 318–340, and 360–370 suggests these regions may be involved in PtdIns4P binding and PtdIns(4,5)P2 feedback inhibition. Positively charged residues are essential for PtdIns(4,5)P2 binding to pleckstrin homology domains, phospholipase C, and actin binding proteins (5, 6, 34–36).

Based on their kinase homology domains minus the insert region, a rooted tree of the PIP5Ks was generated to better understand the relationships within this gene family (Fig. 2D). The mammalian isoforms formed a cluster distantly related to the Fab1-like protein C05E7.5 (32), and PIP5KIa (25), the isoform most closely related to yeast Mss4p, Fab1p. Within this cluster, PIP5KIa forms a subfamily distinct from PIP5KIa, the isofrom most closely related to yeast Mss4p.

**Northern Blotting of PIP5KI**—Northern analysis revealed that PIP5KIa is a widely distributed 4.2-kb transcript that was most highly expressed in skeletal muscle (Fig. 3). High levels of PIP5KIa were also observed in heart, placenta, kidney, and pancreas. Only low levels of expression were seen in brain, liver, and lung. A hybridization signal seen in heart around 3.0 kb could have been cross-hybridization with a 5.4-kb transcript appeared in brain and skeletal muscle.4 Potential explanations for this large transcript include other isoforms such as the 90-kDa or 110-kDa PIP5KIa seen in brain and kidney, with the exception of liver, it implied that they have functions applicable to many cell types. Within each tissue though, the level of expression of each isoform often differed considerably.

**Recombinant PIP5KIa Is Both Active and Immunocross-reactive with Erythroid PIP5KI Antibodies**—The PIP5KIa cDNA was subcloned into the PET28b expression vector to create the hexahistidine-tagged His-PIP5KIa fusion protein. When expressed in E. coli BL21 (DE3), His-PIP5KIa was a 70-kDa protein not observed in control cell lysates (Fig. 4A). The observed protein size is larger than the calculated mass of 65.8 kDa, which includes 3.7 kDa from the fusion protein tag. The 70-kDa recombinant and 68-kDa native erythroid isoforms in a protein unrelated to PIP5KI.

**PIP5KIβ is also present in most tissues, but its distribution was different than that of PIP5KIa. When the same membrane was probed for PIP5KIβ, a major transcript of 3.0 kb was present in all tissues except liver (Fig. 3). It was very highly expressed in heart and moderately expressed in brain and pancreas. In heart, additional hybridization signals were seen of 4.2 and 2.0 kb. Because both PIP5KIa were expressed in all tissues examined, with the exception of liver, it implied that they have functions applicable to many cell types. Within each tissue though, the level of expression of each isoform often differed considerably.

**Fig. 2. Alignment of PIP5KIa and PIP5KIβ with other PIP5K family members.** A, schematic representation of the PIP5K family members: human brain PIP5KI1 and PIP5KI1 STM7 (30), human placenta PIP5KIa (25), C. elegans Fab1-like protein C05E7.5 (32), and S. cerevisiae Mss4p (38) and Fab1p (33). The conserved core common to the PIP5K family members is termed the kinase homology domain (dark shading) (25). Except in Fab1p and its C. elegans homologue, this domain is interrupted by the less conserved insert region. The length of each translated region is given in amino acids above its C terminus. Because of the large size of Mss4p, Fab1p, and C05E7.5, only their C termini containing the kinase homology domains are shown. B, aligned kinase homology domains of PIP5KIs (residues 99–432), PIP5KIβ/STM7 (56–391), PIP5KIa (65–401), Mss4p (414–752), Fab1p (1977–2262), and C05E7.5 (891–1169). Highly conserved residues are shown in black and the insert region in white. The alignment was produced using the Pileup program of GCG. The accession numbers for STM7, PIP5KIa, Mss4p, Fab1p, and C05E7.5 are X92493, U14957, D13716, U01017, and Z67879, respectively. C, amino acid identity between the kinase homology domains, minus the insert region, of the PIP5Ks shown in B. The table was produced by pairwise comparison of the sequences using the Gap program of GCG. D, based on the above alignment and ignoring the insert region, a rooted phylogenetic tree was generated using the Kimura protein distance algorithm and UPGMA method of tree building as implemented in GCG.
kinase activity. Recombinant PIP5Kια was active under the same reaction conditions used for erythroid PIP5Kι. Similarly expressed PIP5Kιβ1 also had PIP5K activity.

Mammalian PIP5Kιs, but not PIP5KII, are stimulated 8–70-fold by phosphatidic acid, depending upon assay conditions and the kinase preparation (11, 13). Thus, the ability of phosphatidic acid to activate the partially purified His-PIP5Kια was examined. Under these assay conditions, no more than a 3-fold stimulation was seen with phosphatidic acid6, suggesting that the E. coli-expressed enzyme may not possess requisite features for activation such as posttranslational modifications, cofactors, or oligomerization.

The regulation and cellular roles of the PIP5Kι and PIP5KII isoforms are clearly different (9). Isolation of the first PIP5Kι cDNAs has shown them to be distinct members of the same lipid kinase family as PIP5KIIι and permits comparative studies of these isoforms. With their cloning, the roles of PIP5Kιs in regulated secretion, cytoskeletal dynamics, and signaling cascades, especially those using the Rho subfamily of small G-proteins, can be further elucidated.

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