Phosphatidylinositol-4-phosphate 5-Kinase Isozymes Catalyze the Synthesis of 3-Phosphate-containing Phosphatidylinositol Signaling Molecules*

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Phosphatidylinositol-4-phosphate 5-kinases (PIP5Ks) utilize phosphatidylinositols containing D-3-position phosphates as substrates to form phosphatidylinositol 3,4-bisphosphate. In addition, type I PIP5K isoforms phosphorylate phosphatidylinositol 3,4-bisphosphate to phosphatidylinositol 3,4,5-trisphosphate, while type II kinases have less activity toward this substrate. Remarkably, these kinases can convert phosphatidylinositol 3-phosphate to phosphatidylinositol 4-phosphate in a concerted reaction. Kinase activities toward the D-3-position of phosphoinositides are comparable with those seen with phosphatidylinositol 4-phosphate as the substrate. Therefore, the PIP5Ks can synthesize phosphatidylinositol 4,5-bisphosphate and two 3-phosphate-containing polyphosphoinositides. These unexpected activities position the PIP5Ks as potential participants in the generation of all polyphosphoinositol signaling molecules.

Two distinct pathways have been characterized for agonist-stimulated signal transduction involving phosphatidylinositol (PtdIns). One pathway entails activation of phosphatidylinositol-specific phospholipase C by extracellular agonists resulting in the hydrolysis of phosphoinositides to generate soluble inositol phosphates including inositol 1,4,5-trisphosphate and diacylglycerol (reviewed in Refs. 1 and 2). The other pathway involves receptor-mediated activation of phosphatidylinositol 3-kinase (PtdIns 3-kinase) to produce the second messengers, phosphatidylinositol 3,4-bisphosphate (PtdIns 3,4-P$_2$) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P$_3$) (reviewed in Refs. 3 and 4).

A pathway for the formation of D-3-phosphatidylinositols, proposed based on kinetic studies of intact human neutrophils, is through phosphorylation of the D-3 position of the myo-inositol ring of phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P$_2$) by a PtdIns 4,5-P$_2$ 3-kinase and subsequent dephosphorylation of PtdIns 3,4,5-P$_3$ to produce a PtdIns 3,4-P$_2$ (5). This pathway has been supported by the existence of the extensively characterized PtdIns 3-kinase enzyme family, which can catalyze in vitro phosphorylation of phosphatidylinositol 4-phosphate (PtdIns 4-P) and PtdIns 4,5-P$_2$. Evidence for a different pathway for the formation of D-3-phosphatidylinositols has been found in human platelets, NIH 3T3 cells, and plants in which phosphorylation of the D-3-position of PtdIns 3-P is followed by phosphorylation of the D-4-position to give PtdIns 3,4-P$_2$ and then of the D-5-position to form PtdIns 3,4,5-P$_3$ (6–10). The importance of these various routes of synthesis has been disputed. Indeed, until now, enzymes that catalyze the direct phosphorylation of PtdIns 3-P and PtdIns 3,4-P$_2$ have not been clearly identified.

Several phosphatidylinositol-4-phosphate 5-kinases (PIP5Ks) have been discovered and characterized as enzymes synthesizing PtdIns 4,5-P$_2$ (reviewed in Ref. 11). The best characterized isoforms are the type I and type II kinases that differ both biochemically and immunologically (12–14). The sequence of a type II PIP5K (PIP5KIa) has established that the PIP5K enzymes belong to a novel family (15). More recently, cDNAs encoding type I PIP5K (PIP5KI) and an additional PIP5KI isoform have been isolated (16–19). The translated sequences of PIP5KI and PIP5KIi enzymes have only 35% amino acid identity in their kinase homology domains, further establishing the distinctiveness of these two subfamilies (16). The PIP5KIa cDNA is identical to a product of the gene reported to be mutated in Friedreich's ataxia, a common hereditary autosomal recessive disease (16, 20, 21). All four recombinant type I and II PIP5Ks have PtdIns 4-P 5-kinase activity (15–17, 19, 21).

None of the PIP5K isoforms have been examined for alternative substrates except phosphatidylinositol for which there was no detectable activity (12–14). We report here that the type I and II PIP5K isoforms also utilize the 3-phosphate-containing phosphatidylinositols, forming PtdIns 3,4-P$_2$ and PtdIns 3,4,5-P$_3$. This supports the existence of an additional pathway for the synthesis of 3-phosphate-containing phosphatidylinositol polyphosphates.
PtdInsP 4/5-Kinasen Phosphorylate D-3-phosphatidylinositol

EXPERIMENTAL PROCEDURES

Materials—PtdIns and PtdIns 4-P were from Boehringer Mannheim or Sigma. PtdIns 3-P and PtdIns 3,4-P2 dipalmitoyl esters were synthesized (22) according to Chen and Prestwich,2 and Thum et al. (24), respectively. PtdIns 3-P and PtdIns 3,4-P2, dipalmitoyl esters were also from Matreya. [3H]inositol 1-phosphate, [3H]Ins 1,4-P2, [3H]Ins 1,3,4-P3 and [γ-32P]ATP were from NEN Life Science Products. Lipofectamine, OPTI-MEM, and fetal bovine serum were obtained from Life Technologies, Inc. All other chemicals were purchased from Sigma.

Expression and Purification of Recombinant PIP5K Isozymeses—Recombinant human PIP5Ks were expressed in Escherichia coli and purified by Ni2+/chelate chromatography (15, 16, 19). These proteins were stored at ϸ10 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.01% sodium azide, and 20% glycerol for the type I isozymes or 50 mM Tris, pH 8.0, and 20% glycerol for the type II isozymes. Polymerase chain reaction-based cloning of the recombinant PIP5K1b used in these experiments resulted in two changes from the published sequence: glutamine 300 to arginine and serine 421 to proline. Both changes lie outside of the conserved kinase homology domain (16). The activity of PIP5K1b is less stable; thus, larger amounts were used to achieve comparable activity in the kinase assays.

Phosphatidylinositol Phosphate Kinase Activity Assay—PIP5K isozymes were assayed in 50-μl reactions containing 50 μM Tris, pH 7.6, 10 mM MgCl2, 0.5 mM EGTA, 2–100 μM substrate prepared in isotonic KCl solution, and 50 μM ATP (4 μCi/ml). The reactions, at 37 or 22 °C, were stopped at 5–40 min. Immunoprecipitate kinase activities were tested for 10.5 min at 22 °C in 50 mM Tris, pH 7.5, 10 mM MgCl2, 1 mM EGTA, 50 μM substrates prepared in Tris buffer and 50 μM ATP (4 μCi/ml). The reactions were stopped by the addition of 100 μl of 1 N HCl, and the lipid products were extracted using 200 μl of chloroform/methanol (1:1). The organic phase was washed at least once in 80 mM 3,4,5-P3 Standards—[32P]3,4,5-P3, [32P]Ins 1,4-P2, and [32P]Ins 1,3,4-P3 were prepared using a recombinant mutant p110 subunit of PtdIns 3-kinase that is constitutively active (26) (gift from L. T. Williams, Chiron). The SF9 cell-expressed p110 mutant PtdIns 3-kinase was purified using the nickel-nitrolotriacetic acid affinity purification method (15, 16, 19). These proteins were conserved kinase homology domain (16). The activity of PIP5KII was assayed at 37 °C. The lipid and protein substrates used were PtdIns 3-P, PtdIns 3,4-P2, or PtdIns 4-P for 10.5 min at 22 °C. The enzyme assays were assayed using 80 μM PtdIns 3-P, PtdIns 3,4-P2, or PtdIns 4-P for 10.5 min at 22 °C. The positions of products of the reaction are marked by arrows. All lanes were from the same TLC plate with different autoradiograph exposures. Exposures were for 5 min (lanes 3) or 15 min (lanes 1, 2, and 9–11) at room temperature or 1.5 h at –80 °C (lanes 4–8 and 12). B, time course of PIP5KII activity. The kinase activity of PIP5KII (2.4 μg) toward 80 μM PtdIns 3-P (filled circles) or PtdIns 4-P (open squares) was assayed for 5–40 min at 37 °C. C, time course of PIP5KII activity. The kinase activity of PIP5KII (0.4 μg) toward 5 μM PtdIns 3-P (filled circles) or PtdIns 4-P (open squares) was determined for 0–20 min at 37 °C.

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FIG. 1. Recombinant PIP5K isozymes phosphorylate 3-phosphate-containing phosphatidylinositol. A, the activities of E. coli-expressed recombinant PIP5K isozymes were assayed using 80 μM PtdIns 3-P, PtdIns 3,4-P2, or PtdIns 4-P for 10.5 min at 22 °C. The enzymes assayed were PIP5K1α (0.2 μg), PIP5K1β (0.7 μg), PIP5KIIα (2 μg), and PIP5KIIβ (180 μg). The positions of products of the reaction are marked by arrows. All lanes were from the same TLC plate with different autoradiograph exposures. Exposures were for 5 min (lanes 3) or 15 min (lanes 1, 2, and 9–11) at room temperature or 1.5 h at –80 °C (lanes 4–8 and 12). B, time course of PIP5KII activity. The kinase activity of PIP5KII (2.4 μg) toward 80 μM PtdIns 3-P (filled circles) or PtdIns 4-P (open squares) was assayed for 5–40 min at 37 °C. C, time course of PIP5KII activity. The kinase activity of PIP5KII (0.4 μg) toward 5 μM PtdIns 3-P (filled circles) or PtdIns 4-P (open squares) was determined for 0–20 min at 37 °C.
enzymes also have activity toward PtdIns 3,4-P2, producing a product that migrates as PtdInsP 3 (Fig. 1A, lanes 1–3) and PtdInsP 4 (Fig. 1A, lanes 3 and 4). In a short reaction (5 min), comparison of PIP5KIIa kinase activity toward PtdIns 3-P and PtdIns 4-P at various concentrations indicated that the activity toward both substrates was dependent on their concentrations (data not shown). The kinetic parameters for PIP5KIIa enzymatic phosphorylation of PtdIns 3-P and PtdIns 4-P are shown in Table I.

The products of the PIP5K reactions using PtdIns 3-P and PtdIns 4-P as substrates were identified by HPLC analysis (Fig. 2). The elution of the internal standards marked by arrows was identical in each pair of runs. A, the deacylated PtdInsP2 product of PIP5KIIa using PtdIns 3-P as a substrate. B, the deacylated PtdInsP2 product of PIP5KIIβ using PtdIns 3,4-P2 as substrate. Different Partisil 10 Sax columns were used for A and B, so the elution positions of the internal standards were different.

![Fig. 2. HPLC analysis of the deacylated glycerophosphorylinositol products of PIP5K reactions.](image)

The apparent Vm value of PIP5KIIa for PtdIns 3-P is 3-fold greater than that of its previously identified substrate PtdIns 4-P (Fig. 1B). In addition, the phosphorylation of PtdIns 3-P was linear for a longer time interval compared with PtdIns 4-P (Fig. 1B). The deacylated products of PIP5KIIa and PIP5KIIβ enzymes using PtdIns 3-P are lower than that of PIP5KIIa toward PtdIns 4-P. As a result, the catalytic efficiency (Vm/Km) for these two substrates is the same. Little activity toward PtdIns 3,4-P2 was detected with the type II PIP5Ks (Fig. 1A, lanes 3 and 4).

The kinetic parameters of PIP5K isozymes with their different substrates are listed in Table I. The time dependence of PIP5KIIa activity using PtdIns 3-P and PtdIns 4-P is shown in Fig. 1C. The substrate preferences of the type I isozymes are different from the type II isozyme in that the type I PIP5K (α and β) enzymes phosphorylate PtdIns 4-P with a much greater Vm than PtdIns 3-P. However, the Km values of the PIP5KIIa enzymes using PtdIns 3-P as a substrate were lower than that of PIP5KIIb.

The ability of four different PIP5K isozymes to phosphorylate PtdIns 3-P, PtdIns 3,4-P2, and PtdIns 4-P is shown in Fig. 1A. When analyzed by TLC, the PtdIns 3-P phosphorylation product migrated as a phosphatidylinositol bisphosphatase (PtdInsP2). The product migrated more slowly than PtdIns 4-P, given the specificity of the PIP5K enzymes toward PtdIns 4-P. Surprisingly, HPLC analysis demonstrated that the deacylated product of all of the PIP5K isozymes using PtdIns 3-P as substrate co-chromatographed with GroPIns 3,4,5-P3. Treatment of the

![Image](image)

**Table I. Kinetic parameters of PIP5K isozymes.**

| Enzymes     | Substrates      | Km (mM) | Vm (pmol/min/mg protein) | Vm/Km (pmol/min/mg protein) |
|-------------|-----------------|---------|--------------------------|-----------------------------|
| PIP5KIIa    | PtdIns 3-P       | 120     | 50                       | 0.8                         |
| PIP5KIIa    | PtdIns 4-P       | 90      | 39                       | 0.8                         |
| PIP5KIIa    | PtdIns 3,4-P2    | 262     | 47                       | 0.8                         |
| PIP5KIIa    | PtdIns 4-P       | 86.2    | 47                       | 0.8                         |
| PIP5KIIa    | PtdIns 3,4-P2    | 29653   | 61.0                     | 5.5                         |
| PIP5KIIb    | PtdIns 3-P       | 65      | 47                       | 2.9                         |
| PIP5KIIb    | PtdIns 4-P       | 1903    | 47                       | 2.9                         |
| PIP5KIIb    | PtdIns 3,4-P2    | 2892    | 72                       | 2.5                         |
| PIP5KIIb    | PtdIns 4-P       | 47      | 47                       | 2.5                         |
| PIP5KIIb    | PtdIns 3,4-P2    | 29653   | 61.0                     | 5.5                         |

† ND, none detected.

**Putative Friedrich’s ataxia gene product.**
putative PtdIns 3,4-P2 products of PIP5K reactions with recombinant 4-phosphatase resulted in release of 32P-labeled inorganic phosphate, confirming that this was PtdIns 3,4-P2 labeled in the D-4-position (data not shown). In addition, treatment of the PtdInsP3 product with the Lowe oculocerebrorenal syndrome 5-phosphatase, an enzyme that specifically hydrolyzes the 5-position phosphate of PtdIns 3,4,5-P3 (29, 30), released 32P-labeled inorganic phosphate (data not shown). This result confirms that the product of this reaction was PtdIns 3,4,5-P3 labeled on the D-5-position.

Reactions using PtdIns 3-P as substrate also contained a product that migrated as PtdIns 3,4,5-P3. This product was observed using PIP5KI isozymes and PIP5KIIα but not PIP5KIIβ (Fig. 1A, lanes 1–4). HPLC analysis confirmed that this was PtdIns 3,4,5-P3, which comigrated with lesser concentrations of lyso-PtdIns 3,4-P2 (20% for PIP5KIs, 45% for PIP5KIIα). The amounts of PtdIns 3,4,5-P3 formed are shown in Table II. Because the substrate concentrations were 80 μM and the intermediate PtdIns 3,4-P2 product was nanomolar where PtdIns 3-P was the substrate, the amount of PtdIns 3,4,5-P3 formed is remarkable. Indeed, the amount of PtdIns 3,4,5-P3 formed from PtdIns 3-P using either PIP5KIα or PIP5KIIα was similar to that using 80 μM PtdIns 3,4-P2 with PIP5KIα. These results suggest that synthesis of PtdIns 3,4,5-P3 from PtdIns 3-P is a concerted reaction. In the case of the type II enzymes, PtdIns 3,4-P2 was not detectably phosphorylated by these enzymes. Yet, when type II kinases use PtdIns 3-P as substrate, PtdIns 3,4,5-P3 is produced.

These data were obtained using recombinant, E. coli-expressed PIP5K isoforms, but similar results were observed using native PIP5Ks from mammalian cells. PIP5KII purified from erythrocytes had similar activity to the PIP5KIIα presented above (data not shown). The ability of PIP5KIα to phosphorylate the 3-phosphate-containing lipids was validated by

![Image](image_url)
immunoprecipitation of the kinase from COS-7 cells. When COS-7 cell lysates were Western blotted with anti-PIP5Kα antibody, a single 68-kDa protein was detected, which was immunoprecipitated with the same antibody (Fig. 3A). The PIP5Kα was not immunoprecipitated using an IgG depleted of PIP5Kα reactivity (Fig. 3A) or preimmune IgG (data not shown). The native PIP5Kα was able to phosphorylate both PtdIns 3-P and PtdIns 3,4-P₂, and the activity toward the former was only 4-fold lower compared with PtdIns 4-P kinase activity. As shown for both PtdIns 3-P and PtdIns 3,4-P₂ production, the concerted reaction could explain why PtdIns 3,4,5-P₃ appears first. However, these arguments were based upon the observation that this PtdIns 3-kinase will phosphorylate all phosphoinositides lacking a phosphate in the D-3-position and that PtdIns 3,4,5-P₃ appears to be the initial product. With the data presented here, an alternative pathway in which PtdIns is phosphorylated by PtdIns 3-kinase and then phosphorylated by a PIP5K isoform is plausible, and the concerted reaction could explain why PtdIns 3,4,5-P₃ appears first.

The PIP5Ks have the potential to produce three signaling molecules: PtdIns 4,5-P₂, PtdIns 3,4-P₂, and PtdIns 3,4,5-P₃. It will be very interesting to determine if the substrate preferences of these PIP5K isoforms are altered by receptor activation or different regulators such as the small G-proteins Rac and Rho (23, 32, 33). It is conceivable that modulation of these activities will be both spatially and temporally regulated, and thus the PIP5K enzymes could participate in a plethora of cellular events by generating multiple messengers. Given the expanded substrate repertoire of these enzymes, we propose that they be designated as phosphatidylinositol phosphatase 4/5-kinases.

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