Type I Phosphatidylinositol-4-phosphate 5-Kinases Synthesize the Novel Lipids Phosphatidylinositol 3,5-Bisphosphate and Phosphatidylinositol 5-Phosphate

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Inositol phospholipids regulate a variety of cellular processes including proliferation, survival, vesicular trafficking, and cytoskeletal organization. Recently, two novel phosphoinositides, phosphatidylinositol 3,5-bisphosphate (PtdIns-3,5-P2) and phosphatidylinositol 3-phosphate (PtdIns-3-P), have been shown to exist in cells. PtdIns-3,5-P2, which is regulated by osmotic stress, appears to be synthesized by phosphorylation of PtdIns-3-P at the D-5 position. No evidence yet exists for how PtdIns-5-P is produced in cells. Understanding the regulation of synthesis of these molecules will be important for identifying their function in cellular signaling. To determine the pathway by which PtdIns-3,5-P2 and PtdIns-5-P might be synthesized, we tested the ability of the recently cloned type I PtdIns-4-P 5-kinases (PIPK5ks) α and β to phosphorylate PtdIns-3-P and PtdIns at the D-5 position of the inositol ring. We found that the type I PIP5Ks phosphorylate PtdIns-3-P to form PtdIns-3,5-P2, the identity of the PtdIns-3,5-P2 product was determined by anion exchange high performance liquid chromatography analysis and periodate treatment. PtdIns-3,4-P2 and PtdIns-3,4,5-P3 were also produced from PtdIns-3-P phosphorylation by both isoforms. When expressed in mammalian cells, PIP5K Iα and PIP5K Iβ differed in their ability to synthesize PtdIns-3,5-P2 relative to PtdIns-3,4-P2. We also found that the type I PIP5Ks phosphorylate PtdIns to produce PtdIns-3-P and phosphorylate PtdIns-3,4-P2 to produce PtdIns-3,4,5-P3. Our findings suggest that type I PIP5Ks synthesize the novel phospholipids PtdIns-3,5-P2 and PtdIns-5-P. The ability of PIP5Ks to produce multiple signaling molecules indicates that they may participate in a variety of cellular processes.

Although phosphoinositides represent only a small fraction of the total cellular lipids, they play critical roles as intracellular signaling molecules. Phosphoinositides regulate a variety of cellular processes including proliferation, survival, vesicular trafficking, and cytoskeletal organization (1). The levels of phosphoinositides in the cell are modulated by external stimuli which regulate the activities of phosphoinositide kinases, phosphatases, and lipases. Several of these enzymes have recently been identified and cloned (1–5), and this work has facilitated the understanding of how phosphoinositides are regulated and what roles they play in cellular signaling. It is now recognized that PtdIns-4,5-P2 has intrinsic signaling capabilities, in addition to its role as a substrate for diacylglycerol, inositol-1,4,5-trisphosphate, and PtdIns-3,4,5-P3 synthesis (3). PtdIns-4,5-P2 binds to pleckstrin homology domains (6–8) and plays a role in exocytosis (9–11). It also regulates the activity of several actin-binding proteins (12). This finding, in combination with the evidence that Rho family small G proteins associate with type I PIP5Ks which synthesize PtdIns-4,5-P2 (13–15), suggests that PtdIns-4,5-P2 may participate in Rho family-mediated actin cytoskeletal rearrangements. As mentioned, PtdIns-4,5-P2 is synthesized by the type I PIP5Ks which phosphorylate PtdIns-4-P on the D-5 position of the inositol ring. Rameh et al. (16) recently discovered an alternative pathway for synthesis, demonstrating that type II PIPKs produce PtdIns-4,5-P2 by phosphorylating PtdIns-5-P on the D-4 position of the inositol ring. Although this work also provided evidence for the existence of PtdIns-5-P in cells, it remains unclear how this lipid is synthesized.

The D-3 phosphoinositides, PtdIns-3-P, PtdIns-3,4-P2 and PtdIns-3,4,5-P3 also have distinct roles in cellular signaling. PtdIns-3-P, which is present constitutively in cells, plays a role in Golgi to vacuole trafficking in yeast (17). PtdIns-3,4-P2 and PtdIns-3,4,5-P3, which accumulate rapidly after cell stimulation in contrast to PtdIns-3-P, interact directly with pleckstrin homology domains (7, 8, 18–20) and Src homology 2 domains (21). The binding of PtdIns-3,4-P2 and PtdIns-3,5-P2 to these domains likely results in the recruitment to the membrane of multiple signaling proteins which could initiate secondary signaling cascades. PtdIns-3,4-P2 and PtdIns-3,5-P2 have also been shown to activate the serine/threonine kinase, Akt, its upstream kinase, PDK-1, and some protein kinase C isoforms (22–24). A number

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†† The abbreviations used are: PtdIns-4,5-P2, phosphatidylinositol 4,5-bisphosphate; PtdIns, phosphatidylinositol; PtdIns-3-P, phosphatidylinositol 3-phosphate; PtdIns-4-P, phosphatidylinositol 4-phosphate; PtdIns-5-P, phosphatidylinositol 5-phosphate; PtdIns-3,4-P1, phosphatidylinositol 3,4-bisphosphate; PtdIns-3,5-P2, phosphatidylinositol 3,5-bisphosphate; PtdIns-3,4,5-P3, phosphatidylinositol 3,4,5-trisphosphate; Ins-1,3,4,5-P4, inositol 1,3,4,5-tetrakisphosphate; GroPIns, glycerophosphorylinositol; type I PIP5K, type I phosphatidylinositol-4-phosphate 5-kinase; type II PIPK, type II phosphatidylinositol phosphate kinase; P38K, phosphoinositide 3-kinase; HPLC, high performance liquid chromatography.
of PI3K family members have now been identified which can catalyze the *in vitro* phosphorylation of PtdIns, PtdIns-4-P, and/or PtdIns-4,5-P$_2$ on the D-3 position of the inositol ring (25). Kinetic studies in $^{32}$PO$_4$-labeled cells suggest that PtdIns-3,4-P$_2$, and PtdIns-3,5-P$_2$ are synthesized by the phosphorylation of PtdIns-4,5-P$_2$ on the D-3 position by PI3K to produce PtdIns-3,4,5-P$_3$, followed by a dephosphorylation of PtdIns-3,4,5-P$_3$ on the D-5 position by a phosphatase to form PtdIns-3,4-P$_2$ (26). Studies in platelets provide evidence for an alternative synthetic pathway for D-3 phosphoinositide in which PtdIns is first phosphorylated on the D-3 position to produce PtdIns-3-P, followed by the phosphorylation of the D-4 position to form PtdIns-3,4-P$_2$ (27–29).

A newly discovered phosphoinositide in the PI3K pathway has recently been described. Analysis of phosphoinositides present in smooth muscle cells revealed an inositol containing lipid with chromatographic properties similar to PtdIns-3,4,5-P$_3$, which we proposed to be PtdIns-3,5-P$_2$ (30). Whiteford et al. (31) recently verified that PtdIns-3,5-P$_2$ is present in fibroblasts. Dov et al. (32) also found PtdIns-3,5-P$_2$ in *Saccharomyces cerevisiae* and showed that the level of this lipid is regulated by osmotic strength changes (32). The synthesis of PtdIns-3,5-P$_2$ is blocked by the PI3K inhibitor wortmannin (31). Analysis of the specific activity of the D-3 and D-5 phosphates indicates that PtdIns-3,5-P$_2$ is synthesized primarily by phosphorylation of PtdIns-3-P at the D-5 position (31, 32). The specific activity data and the fact that PtdInsP3 is absent in cells under conditions where PtdIns-3,5-P$_2$ is found argue that a phosphatase is unlikely to be involved in PtdIns-3,5-P$_2$ synthesis.

The only known kinases that phosphorylate the D-5 position of the inositol ring are the type I PIP5Ks. As mentioned previously, the type II PIP5Ks have recently been demonstrated to phosphorylate the D-4 position of the inositol ring (16). While the function of PtdIns-3,5-P$_2$ in the cell is unknown, we were interested in the pathway by which it is synthesized and tested whether type I PIP5Ks can phosphorylate the D-5 position of PtdIns-3-P to form PtdIns-3,5-P$_2$. We find that type I PIP5Ks do phosphorylate PtdIns3-P at the D-5 position. We also find that these kinases can produce PtdIns-5-P from PtdIns and can synthesize PtdInsP3 from either PtdIns-3-P or PtdIns-3,4-P$_2$.

**EXPERIMENTAL PROCEDURES**

**Materials**—PtdIns and PtdIns-4-P were obtained from Avanti Polar Lipids Inc. and Sigma, respectively. Synthetic dipalmitoyl PtdIns-3-P, PtdIns-3,4-P$_2$, and PtdIns-3,5-P$_2$ were synthesized as described (34). $[^3H]$PtdIns-4-P, $[^3H]$PtdIns-3,4-P$_2$, $[^3H]$Ins-3,4,5-P$_3$, $[^3H]$Ins-1,4,5-P$_3$, and $[^3H]$Ins-3,4,5-P$_3$ were purchased from NEN Life Science Products. LipofectAMINE and Opti-MEM were obtained from Life Technologies, Inc. All other chemicals were from Sigma.

*Plasmids*—N-terminal HA-tagged murine cDNAs of type I PIP5K and type II PIP5K were generated as described previously (5). The cDNAs of the PIP5Ks lacking the HA-tag were subcloned into the pCMV and pEGFP plasmid vectors to create the type I PIP5K and type II PIP5K expression plasmids, respectively.

Expression and Purification of Recombinant PIP5Ks Isoforms—Glutathione S-transferase fusion proteins of the type I PIP5Ks were expressed in *Escherichia coli* and purified with glutathione-Sepharose 4B beads as described previously (15). The glutathione-Sepharose beads with 50 ml Tris buffer, pH 8.0, containing 20 ml reduced glutathione and then concentrated in a Centricon filter after several washes with buffer (50 ml Tris, pH 7.5, 150 ml NaCl, 1 ml MgCl$_2$, 5 ml dithiothreitol). Glycerol was added to the proteins before storing at $-80^\circ$ C.

**Cell Culture and Transfections**—293 E1A cells were maintained in Dulbecco's modified medium containing 10% heat inactivated fetal calf serum. Cells were transfected with LipofectAMINE using 4 ml of HA-tagged PIP5Ks per 10-cm plate. Cells were harvested 18 h after transfection.

**Immunoprecipitation of PIP5Ks from 293 E1A Cells**—Cells were rinsed with phosphate-buffered saline and then lysed in 600 ml of lysis buffer (50 mm Tris, pH 7.5, 50 mm NaCl, 5 mm MgCl$_2$, 1% Nonident P-40, 10% glycerol, 1 mm dithiothreitol, 4 g/ml each leupeptin and pepstatin, and 200 ml ABE SF). The clarified lysates were incubated with 1.5 ml of an anti-HA antibody (12CA5 from Boehringer Mannheim) and protein A-Sepharose beads for 3 h at 4°C. The beads were then washed twice with lysis buffer and twice with TMN (50 mm Tris, pH 7.5, 50 mm NaCl, and 5 mm MgCl$_2$).
5 molar to 1% (NH₄)₂HPO₄ over 40 ml, 3 to 10% 1 M (NH₄)₂HPO₄ over 25 ml, and 13 to 65% 1 M (NH₄)₂HPO₄ over 25 ml. Eluate from the HPLC column flowed into an on-line continuous flow scintillation detector (Radiomatic Instruments, FL) for isotope detection.

Periodate Oxidation—Periodate oxidation was performed as described with the following modifications (36). Deacylated lipids were incubated in the dark at 25 °C with 10 or 100 mM periodic acid, pH 4.5, for 30 min or 36 h, respectively. The remaining oxidizing reagent was removed by adding 500 mM ethylene glycol and incubating the reaction in the dark for 30 min. 2% 1,1-dimethylhydrazine, pH 4.5, was then added to a final concentration of 1% and the reaction was allowed to proceed for 4 h at 25 °C. The mixture was then purified by ion exchange using a Dowex 50W-X8 cation-exchange resin (20–50 mesh, acidic form), dried, and applied to an HPLC column. PtdIns-3,4-P₂ and PtdIns-3,4,5-P₃ controls used in this experiment were prepared using recombinant Sf9 cell-expressed PI3K.

RESULTS

PIP5Ks Phosphorylate PtdIns, PtdIns-3-P, and PtdIns-3,4-P₂—The recent description of the presence of PtdIns-3,5-P₂ in fibroblasts rekindled our interest in this phosphoinositide and how it is produced. Since it was recently shown that type II PIPK phosphorylates the D-4 position of PtdIns-5-P (16), the synthesis of PtdIns-3,5-P₂ must either be catalyzed by a type I PIP5K, a phosphatase, or a new enzyme. To further define the substrates and products of the type I PIP5Ks, we provided PtdIns, PtdIns-3-P, PtdIns-4-P, PtdIns-3,4-P₂, and PtdIns-3,5-P₂ as substrates and analyzed the products. As our enzyme source, we used recombinant PIP5Ks Iα and PIP5K Iβ (according to the nomenclature proposed by Ishihara et al. (5)) that were produced as glutathione S-transferase fusion proteins in bacteria. The products of the phosphorylation reactions were analyzed by TLC using a system that separates PtdInsP, PtdInsP₂, and PtdInsP₃, and visualized using a Molecular Analyst. As expected, the type I PIP5Ks converted PtdIns-4-P to PtdInsP₂ (Fig. 1) (4, 5). Surprisingly, the enzymes were also capable of synthesizing other lipid products. Both PIP5K Iα and PIP5K Iβ phosphorylated PtdIns-3,4-P₂ to produce PtdInsP₃ and phosphorylated PtdIns-3-P to produce PtdInsP₂ and PtdInsP₃ (Fig. 1). PtdIns was also converted to PtdInsP and PtdInsP₂ by the type I PIP5Ks (although bacterially expressed PIP5K Iβ was more active than PIP5K Iα at phosphorylating PtdIns). In contrast, PtdIns-3,5-P₂ was a poor substrate for both enzymes (1/5 as good as PtdIns-3,4-P₂). The PtdInsP₂ controls used in this experiment were prepared using recombinant Sf9 cell-expressed PI3K.

HPLC Analysis of the Products from the Phosphorylation of PtdIns-3-P and PtdIns-3,4-P₂—To determine the identity of the products, they were deacylated and analyzed by anion exchange HPLC and detected using a Radiomatic Instruments, FL, liquid scintillation detector.
Type I PIP5Ks Synthesize PtdIns-3,5-P$_2$ and PtdIns-5-P

change chromatography using HPLC. As expected from previous studies (4, 5), the PtdIns$_2$ produced using PtdIns-4-P as a substrate for either enzyme was exclusively PtdIns-4,5-P$_2$ (not shown). The product of PtdIns-3,4-P$_2$ phosphorylation by either enzyme was confirmed to be PtdIns-3,4,5-P$_3$, in agreement with a recent study by Zhang et al. (Fig. 2B) (33). However, in contrast to the findings of Zhang et al. (33), the deacylated PtdIns$_2$ produced by phosphorylation of synthetic PtdIns-3-P with either type I isoenzyme was resolved into two peaks by HPLC analysis (75.5 and 77 min, Fig. 2A). The peak at 77 min comigrated with the glycerophosphorylinositol 3,4-bisphosphate (GroPIns-3,4-P$_2$) standard while the peak at 75.5 min was resolved into two peaks by HPLC analysis (75.5 and 77 min, Fig. 2A). The peak at 77 min comigrated with the glycerophosphorylinositol 3,4-bisphosphate (GroPIns-3,4-P$_2$) standard while the peak at 75.5 min comigrated with the glycerophosphorylinositol 3,4-bisphosphate (GroPIns-3,4-P$_2$) standard while the peak at 75.5 min comigrated with the glycerophosphorylinositol 3,4-bisphosphate (GroPIns-3,4-P$_2$) standard while the peak at 75.5 min comigrated with the glycerophosphorylinositol 3,4-bisphosphate (GroPIns-3,4-P$_2$) standard while the peak at 75.5 min comigrated with the glycerophosphorylinositol 3,4-bisphosphate (GroPIns-3,4-P$_2$) standard while the peak at 75.5 min comigrated with the glycerophosphorylinositol 3,4-bisphosphate (GroPIns-3,4-P$_2$) standard while the peak at 75.5 min comigrated with the glycerophosphorylinositol 3,4-bisphosphate (GroPIns-3,4-P$_2$) standard while the peak at 75.5 min comigrated with the glycerophosphorylinositol 3,4-bisphosphate (GroPIns-3,4-P$_2$) standard while the peak at 75.5 min comigrated with the 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Zhang et al. (33) did not detect a second peak corresponding to GroPIns-3,5-P₂ when analyzing the reaction products of the phosphorylation of PtdIns-3-P by type I PIP5Ks. The best explanation for this discrepancy is that the anion exchange HPLC analysis done by Zhang et al. (33) did not separate GroPIns-3,4-P₂ from GroPIns-3,5-P₂. We also detected two additional peaks in the reaction products of PtdIns-3-P phosphorylation by type I PIP5Ks (Fig. 2A). The peak at 98 min eluted at the known position for GroPIns-3,4,5-P₃ (Fig. 2A). The peak at 95 min (X) was not identified.

To demonstrate that the PIP5K β isoforms produced shown in Fig. 2A were synthesized from the phosphorylation of PtdIns-3-P and not a contaminating lipid, we analyzed the ability of the type I PIP5Ks to phosphorylate [³²P]PtdIns-3-P which was enzymatically produced by PI3K. Bacterially expressed PIP5K β was incubated with [³²P]PtdIns-3-P and unlabeled ATP for 10 min at room temperature. The lipid products were then extracted and analyzed. We found that PIP5K β converted [³²P]PtdIns-3-P to [³²P]PtdIns-3,5-P₂, [³²P]PtdIns-3,4-P₂, and [³²P]PtdIns-5-P₃ (Fig. 3).

**Type I PIP5Ks Phosphorylate PtdIns-3-P to Produce PtdIns-3,5-P₂**—Although the major PtdInsP₂ product from the phosphorylation of PtdIns-3-P by type I PIP5Ks appeared to be PtdIns-3,5-P₂ based on its migration position, we further investigated its identity using periodate. Since this lipid was not PtdIns-3,4-P₂ or PtdIns-4,5-P₂, it could only be PtdIns-3,5-P₂, PtdIns-2,3-P₂, or PtdIns-3,6-P₂. The latter two products have never been described as existing in vivo, but can be distinguished from PtdIns-3,5-P₂ by their sensitivity to periodate treatment. Of this group, only PtdIns-3,5-P₂ lacks a vicinal diol for periodate attack of the inositol ring and is therefore resistant to cleavage. We treated the products of PtdIns-3,5-P₂ phosphorylation with periodate. GroPIns-3,4,5-P₃ (which is sensitive to periodate) and GroPIns-3,4,5-P₃ (which is resistant to periodate) were also treated as controls. The reaction products were analyzed by anion exchange chromatography using an HPLC.

Short periodate treatment (30 min) resulted in the conversion of the GroPIns-3,4,5-P₃ and GroPIns-3,5,6-P₃ products to InsP₃ and the GroPIns-3-P₂ product to InsP₂ due to cleavage of the glycerol moiety (Fig. 4A). Since PtdIns-3,4,5-P₃ lacks a vicinal diol and is therefore resistant to periodate cleavage (Fig. 4, E and F), we used the InsP₃ derived from the PtdInsP₃ present in our sample as an internal control for yield after prolonged periodate treatment (36 h). Prolonged periodate treatment resulted in the loss of 50% of InsP₃ (Fig. 4B). Similar treatment of Ins-1,3,4,5-P₄ resulted in almost complete loss of radiolabeled material (3% remaining) at the position of Ins-1,3,4,5-P₄ (Fig. 4, C and D). However, under the same conditions, approximately 30% of the GroPInsP₂ product of the type I PIP5K β was recovered at the position expected for Ins-1,3,5-P₃ (Fig. 4B). When corrected for the reaction yield based on the treatment of PtdInsP₃, 60% of the InsP₃ product was resistant to periodate. Similar results were obtained with the PtdInsP₃ phosphorylation products of PIP5K α. These results, in agreement with those in Figs. 2A and 3, indicate that the majority of the PtdInsP₂ produced by the Type I PIP5Ks is PtdIns-3,5-P₂.

Synthesis of either PtdIns-3,4-P₂ or PtdIns-3,5-P₂ in our in vitro assays could have resulted from the dephosphorylation of PtdIns-3,4,5-P₃ by a contaminating phosphatase. To investigate this possibility we provided [³²P]PtdIns-3,4,5-P₃, labeled at the D-3 position by PI3K to both the α- and β-isoforms of type I PIP5K and analyzed the products. We found no [³²P]PtdIns-3,4,5-P₃ produced when the type I PIP5Ks were incubated with [³²P]PtdIns-3,4,5-P₃ (not shown). These results suggest that the type I PIP5Ks can phosphatase the D-4 or the D-5 position of Ptd-Ins-3-P, and that with relatively high frequency both sites become phosphorylated in a concerted fashion.

**Type I PIP5Ks Produce PtdIns-5-P**—The product of the reaction using PtdIns as substrate was also identified by decylation and HPLC chromatography. As shown in Fig. 5, decylated PtdInsP₂ produced using TLC purified PtdIns as the substrate for the E. coli-expressed PIP5K β enzyme migrates distinctly from GroPIns-3-P or GroPIns-4-P at the position of GroPIns-5-P (16). Similar results were obtained using bacterially expressed PIP5K Iso as the enzyme source (data not shown). PtdIns-5-P has been found in cells (16), and this result suggests that it could be synthesized by type I PIP5Ks. A small amount of PtdInsP₂, which was identified as PtdIns-4,5-P₂, was also produced using PtdIns as substrate (not shown). Since the PtdIns used in this experiment was TLC purified to eliminate the possibility that PtdIns-4-P was present as a contaminant, this result suggests that type I PIP5Ks can phosphorylate both the D-4 and D-5 positions of the inositol ring. However, given that PtdIns-5-P is a poor substrate for type I PIP5Ks (16), it is likely that the type I PIP5Ks only synthesize PtdIns-4,5-P₂ from PtdInsP₂ in a concerted reaction at a low rate.

**Substrate Preference of the Type I PIP5Ks**—To investigate the relative substrate preferences, we determined the apparent Kₘ, Vₘₐₓ and Vₘₐₓ/Kₘ ratio for the various substrates of the bacterially expressed type I PIP5Ks. As expected, PtdIns-4-P was the favored substrate of both the α- and β-isoforms. Bacterially expressed PIP5K α, which was significantly less active than PIP5K β, did not efficiently phosphorylate the other phosphoinositides tested. In contrast, PIP5K β phosphorylated PtdIns-3-P about one-sixth as well as PtdIns-4,5-P₂ (Vₘₐₓ/Kₘ ratio of 92 pmol/min/mg compared with 564 pmol/ min/mg). Given the high ratio of PtdIns-4-P to PtdIns-3-P in mammalian cells (approximately 20:1), it is likely that the type I PIP5Ks only synthesize PtdIns-4,5-P₂ from PtdIns in a concerted reaction at a low rate.
and then assayed for their ability to phosphorylate PtdIns-3-P, PtdIns-3,4-P$_2$, and PtdIns. The products of the reactions were deacylated and analyzed by HPLC. The PIP5Ks expressed in 293 cells were both quite active, in contrast to the bacterially produced enzymes where PIP5K I$_b$ was significantly more active than PIP5K I$_a$. Both mammalian expressed type I PIP5K isoforms were able to phosphorylate PtdIns-3-P to produce PtdIns-3,5-P$_2$, PtdIns-3,4,5-P$_3$, and PtdIns-3,4,5-P$_3$ (Fig. 6, A and D). They were also able to phosphorylate PtdIns-3,4-P$_2$ to produce PtdIns-3,4,5-P$_3$ (Fig. 6, B and E), and PtdIns to produce PtdIns-5-P (Fig. 6, C and F). In contrast to the bacterially expressed enzymes, however, the mammalian expressed PIP5Ks differed in their ability to synthesize PtdIns-3,5-P$_2$ relative to PtdIns-3,4-P$_2$. When PIP5K I$_a$ phosphorylated PtdIns-3-P, it produced more PtdIns-3,5-P$_2$ than PtdIns-3,4-P$_2$ (Fig. 6B), whereas PIP5K I$_b$ produced equal or more PtdIns-3,4-P$_2$ than PtdIns-3,5-P$_2$ (Fig. 6E). It is unclear whether this difference between PIP5K I$_a$ and PIP5K I$_b$ is the result of PIP5K I$_a$ being more efficient at synthesizing PtdIns-3,5-P$_2$ or less efficient at producing PtdIns-3,4-P$_2$.

**DISCUSSION**

We have found that the type I PIP5Ks can synthesize the novel phospholipids PtdIns-3,5-P$_2$ and PtdIns-5-P by phospho-

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**FIG. 6.** HPLC analysis of the products from the phosphorylation of PtdIns, PtdIns-3-P, and PtdIns-3,4-P$_2$ by the type I PIP5Ks produced in mammalian cells. 293 E1A cells were transfected with the HA-tagged constructs pEBB-PIP5K I$_a$ and pEBB-PIP5K I$_b$. The PIP5Ks were immunoprecipitated using an anti-HA antibody and then assayed for their ability to phosphorylate PtdIns-3-P (A and D), PtdIns-3,4-P$_2$ (B and E), and PtdIns (C and F). The phosphorylation products were chloroform-extracted, deacylated, and analyzed by HPLC. Products of PIP5K I$_a$ are shown in panels A-C, whereas products of PIP5K I$_b$ are shown in panels D-F. Migration positions of the tritiated standards included in each run are indicated. These data are representative of four experiments.
Type I PIP5Ks Synthesize PtdIns-3,5-P₂ and PtdIns-5-P

D-3 and D-5 phosphates

PtdIns-3-P by phosphorylating PtdIns on the D-3 position. Ptd-

phosphoinositides: PtdIns-3,5-P₂, PtdIns-3,4-P₂, PtdIns-
in vivo

intermediate in the synthesis of PtdIns-3,4,5-P₃ since it is a

D-4 position (16). The existence of PtdIns-3,5-P₂ has only recently been veri-

fied in yeast, mammalian, and plant cells (31, 32). Although present in resting cells, the level of this lipid appears to be

regulated by osmotic strength changes (32). Since PtdIns-

3,5-P₂ is unlikely to be produced by a phosphatase.

produces PtdIns-3-P, followed by the phosphorylation of the

pathway is provided by studies in platelets which demon-
catalyzing this reaction

propose that the type I PIP5Ks are the kinases responsible for

participate in a variety of cellular processes.

receptors and/or proteins known to interact with type I PIP5Ks such as Rho family small GTP-binding proteins (13–15) alter

of the specific activity of the type I PIP5Ks for their various substrates.

REFERENCES
1. Carpenter, C. L., and Cantley, L. C. (1996) Curr. Opin. Cell Biol. 8, 153–158
2. Vanhaesebroeck, B., Leevers, S. J., Panayotou, G., and Waterfield, M. D. (1997) Trends Biochem. Sci. 22, 267–272
3. Loijens, J. C., Boronenkov, I. V., Parker, G. J., and Anderson, R. A. (1996) Adv. Enzyme Regul. 36, 115–140
4. Loijens, J. C., and Anderson, R. A. (1996) J. Biol. Chem. 271, 32937–32943
5. Ishihara, H., Shibasaki, Y., Katagiri, H., Yaziya, Y., Asano, T., and Oka, Y. (1996) J. Biol. Chem. 271, 29611–29614
6. Harlan, J. E., Hajduk, P. J., Yoon, H. S., and Fesik, S. W. (1994) Nature 371, 168–170
7. Salin, K., Bottomley, M. J., Querfurth, E., Zvelebil, M. J., Geut, I., Scaife, R., Margolis, R. L., Gigg, R., Smith, C. I., Driscoll, P. C., Waterfield, M. D., and Panayotou, G. (1996) EMBO J. 15, 6241–6250
8. Rameh, L. E., Arvidsson, A., Carraway, K. L., III, Couvillon, A. D., Ruthbun, G., Gempton, A., Vanhaeuser, G., Czech, M. P., Ravichandran, K. S., Burakoff, S. J., Wang, D.-S., Chen, C.-S., and Cantley, L. C. (1997) J. Biol. Chem. 272, 22059–22066
9. Eberhard, D. A., Cooper, C. L., Low, M. G., and Holz, R. W. (1990) Biochem. J. 268, 15–25
10. Hay, J. C., Fisette, P. L., Jenkins, G. H., Fukami, K., Takenawa, T., Anderson, R. A., and Martin, T. F. J. (1995) Nature 374, 173–177
11. Lisievschi, M., and Cantley, L. C. (1995) Cell 81, 659–662
12. Janney, A. (1994) Annu. Rev. Physiol. 56, 169–191
13. Toliai, K. F., Cantley, L. C., and Carpenter, C. L. (1995) J. Biol. Chem. 270, 17656–17659
14. Ren, X.-D., Bozhe, G. M., Traynor-Kaplan, A., Jenkins, G. H., Anderson, R. A., and Schwartz, M. A. (1996) Mol. Biol. Cell 7, 435–442
15. Toliai, K. F., Couvillon, A. D., Cantley, L. C., and Carpenter, C. L. (1998) Mol. Cell. Biol. 18, 762–770
16. Rameh, L. E., Toliai, K. T., Duckworth, B., and Cantley, L. C. (1997) Nature 390, 192–196
17. Schu, P. V., Takegawa, K., Fry, M. J., Stack, J. H., Waterfield, M. D., and Emr, S. D. (1993) Science 260, 88–91
18. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) Science 275, 665–668
19. Krippel, A., Kavanagh, W. M., Pot, D., and Williams, L. T. (1997) Mol. Cell. Biol. 17, 338–344
20. Freech, M., Andjelkovic, M., Ingleby, E., Reddy, K. K., Falke, J. R., and Hemmings, B. A. (1997) J. Biol. Chem. 272, 8474–8481
21. Rameh, L. E., Chen, C.-S., and Cantley, L. C. (1995) Cell 83, 821–830
22. Toker, A., and Cantley, L. C. (1997) Nature 387, 673–676
23. Stokoe, D., Stephens, L. R., Copeland, T., Gaffney, P. R., Reese, C. B., Painter, G. H. F., Holmes, A. B., McCormick, F., and Hawkins, P. T. (1997) Science 277, 567–570
24. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) Curr. Biol. 7, 261–289
25. Fruman, D. A., Meyers, R. E., and Cantley, L. C. (1998) Annu. Rev. Biochem. 265, in press
26. Stephens, L. R., Hughes, K. T., and Irvine, R. F. (1991) Nature 351, 33–39
27. Yamamoto, K., Graziani, A., Carpenter, C., Cantley, L. C., and Lapetina, E. G. (1990) J. Biol. Chem. 265, 22086–22090
28. Cunningham, T. W., Lips, D. L., Bansal, V. S., Caldwell, K. W., Mitchell, C. A., and Majerus, P. W. (1990) J Biol Chem 265, 21676–21683
29. Kucera, G. L., and Rittenhouse, S. E. (1990) J. Biol. Chem. 265, 3543–3548
30. Auger, K. R., Seropian, L. A., Sohlof, S. P., Libby, P., and Cantley, L. C. (1988) Cell 57, 167–175
31. Whiteford, C. C., Brearley, C. A., and Ulug, E. T. (1997) Biochem. J. 323, 597–601
32. Dove, S. K., Cooke, F. T., Douglas, M. R., Sayers, L. G., Parker, P. J., and Michell, R. H. (1997) Nature 390, 187–192
33. Zhang, X., Loijens, J. C., Boronenkov, I. V., Parker, G. J., Norris, F. A., Chen, J., Thum, O., Prestwich, G. D., Majerus, P. W., and Anderson, R. A. (1997) J. Biol. Chem. 272, 17756–17761
34. Thum, O., Chen, J., and Prestwich, G. D. (1996) Tetrahedron Lett. 37, 9917–9920
35. Seropian, L. A., Auger, K. R., and Cantley, L. C. (1991) Methods Enzymol. 198, 78–87
36. Stephens, L., Hawkins, P. T., Carter, N., Chahwala, S. B., Morris, A. J., Whetton, A. D., and Downes, P. C. (1988) Biochem. J. 249, 271–282