Phosphatidylinositol (PI) 4-kinases catalyze the synthesis of PI 4-phosphate, an important intermediate for the synthesis of membrane polyphosphoinositides, regulators of multiple cellular functions. Two mammalian PI 4-kinases have been cloned, a 230-kDa enzyme (α-form) and a 110-kDa (β-form), both of which are inhibited by >0.1 μM concentrations of the PI 3-kinase inhibitor, wortmannin (WT). In the present study, we created a glutathione S-transferase-PI4Kβ fusion protein for expression in Escherichia coli. The purified protein was biologically active and phosphorylated PI in its 4-position with WT sensitivity and kinetic parameters that were identical to those of purified bovine brain PI4Kβ. In addition to its lipid kinase activity, the enzyme exhibited autophosphorylation that was enhanced by Mn2+ ions and inhibited by WT and another PI 3-kinase inhibitor, LY 294002. The recombinant protein was unable to transphosphorylate, but its isolated C-terminal catalytic domain still displayed autophosphorylation, suggesting that the autophosphorylation site resides within the C-terminal catalytic domain of the protein and is held in position by intramolecular interactions. Autophosphorylation inhibited subsequent lipid kinase activity, which was reversed upon dephosphorylation, by protein phosphatases, PP1 and PP2A1, suggesting that it may represent a regulatory mechanism for the enzyme. Phosphorylation of endogenous or overexpressed PI4Kβ was also observed in COS-7 cells; however, the in vivo phosphorylation of the expressed protein was only partially inhibited by WT and also occurred in a catalytically inactive form of the enzyme, indicating the presence of additional phosphorylation site(s). Successful bacterial expression of PI4Kβ should aid research on the structure-function relationships of this protein as well as of other, structurally related enzymes.

Inositol phospholipids have long been recognized as important regulators of multiple cellular functions. Initially, it was believed that their role was to serve as substrate for the receptor-mediated production of two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol, by their phospholipase C-mediated hydrolysis (1). More recently, it became increasingly evident that phosphatidylinositol 4,5-bisphosphate as well as the 3-phosphorylated inositides can serve additional roles as membrane anchors for the assembly of protein signaling complexes in specific membrane compartments (2).

Inositol phospholipids are produced by sequential phosphorylation of phosphatidylinositol (PI)1 by an increasing number of inositol lipid kinases, including the various classes of PI 3-kinases (3), the PI 4-kinases (4), and the PIP kinases (5, 6). Interestingly, another group of enzymes, termed PI kinase-related kinases, also possess the signature motifs within their ATP-binding catalytic domains that are characteristic for PI kinases. PI kinase-related kinases are serine/threonine kinases with no apparent PI kinase activity (7). In contrast, some of the PI 3-kinases do possess protein kinase activity in addition to being bona fide lipid kinases. For example, PI 3-kinase α phosphorylates its regulatory subunit, p85 (8, 9), and PI 3-kinase δ autophosphorylates its C-terminal tail (10). Both modifications greatly reduce the lipid kinase activity of the respective proteins. Vps34p, a yeast PI 3-kinase involved in protein sorting, and the G protein-activated PI 3-kinase γ also display protein kinase activity and undergo autophosphorylation, although with no apparent change in their lipid kinase activities (11, 12). Intriguingly, a recent study has indicated that the protein kinase activity of PI 3-kinase γ might be more relevant to some of its downstream signaling than its lipid kinase activity (13).

While a number of studies have addressed the question of lipid versus protein kinase activities of PI 3-kinases, relatively little is known about such properties of PI 4-kinases. PI 4-kinases are the enzymes that catalyze the 4-phosphorylation of PI, the first step in a reaction sequence that leads to the formation of most of the polyphosphoinositides. (Recent evidence suggests that some 5-phosphorylation of PI may precede 4-phosphorylation (14)). The majority of the cellular PI 4-kinase activity is represented by the tightly membrane-bound type II 4-kinase enzyme, which has been purified from various tissues as a 50–55-kDa protein (see Ref. 15 for a review) but still awaits molecular cloning and characterization. In contrast, two distinct forms of the less abundant type III PI 4-kinases have been purified from bovine adrenal (16) and brain (17) and cloned from various species including humans (18). These two enzymes, a 110-kDa β-form and a 230-kDa α-form, are homologues of two yeast PI 4-kinases that are products of the PIK1 and STT4 genes, respectively (19, 20). These proteins also contain the characteristic signature of the ATP-binding catalytic domain of PI 3-kinases and are inhibited by the microbial product, WT, the most potent inhibitor of PI 3-kinases (21).

In the present study, we report the bacterial expression of bovine PI4Kβ with catalytic properties similar to those of the purified enzyme. We also show that PI 4-kinase β undergoes

1 The abbreviations used are: PI, phosphatidylinositol; PI4Kα, phosphatidylinositol 4-kinase α; GST, glutathione S-transferase; TPEN, N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine; WT, wortmannin; PAGE, polyacrylamide gel electrophoresis.
autophosphorylation but is unable to transphosphorylate and that its autophosphorylation decreases its lipid kinase activity. It is also demonstrated that PI4Kβ is phosphorylated in vivo in mammalian cells, but the majority of the in vivo phosphorylation is not due to autophosphorylation of the enzyme. Further studies with the recombinant enzyme will help us to better understand the functions and regulation of this enzyme and the role of 4-phosphorylated lipids in cell regulation.

**EXPERIMENTAL PROCEDURES**

**Materials**—[^1]Wortmannin-17-ol (19.7 Ci/mmol) and [γ-32P]ATP (3000–6000 Ci/mmol) were purchased from NEN Life Science Products. Ortho-[32P]phosphate was from ICN Biochemical Research Products (Costa Mesa, CA). Reagents for the cloning and purification of GST-PI 4-kinase β were from Amersham Pharmacia Biotech. ATP, adenosine, and WT were obtained from Sigma. Phosphatidylinositol was purchased from Fluka (Ronkonkoma, NY), and 294002, PP1, PP2A, and okadaic acid were purchased from Calbiochem. Other reagents were of analytical or high pressure liquid chromatography grade.

**Bacterial Expression of PI 4-kinase β**—The 2.4-kilobase pair insert encoding the full-length bovine phosphatidylinositol 4-kinase β was amplified by long-range polymerase chain reaction (primers 5′-gtggctc-cagttgcatgcatggagt-3′ and 5′-aggggcccgtagctggtaggtgctag-3′) using the pCDNAs.1-PI4KIIß (16) as the template. After cutting with the restriction enzymes BamHI/NcoI, the polymerase chain reaction product was ligated into the pGEX-4T-2 or pGEX-6P-3 plasmid (Amersham Pharmacia Biotech) precut with the same enzymes. The inserts of the plasmids were sequenced, and the constructs were transfected into the BL21 strain of E. coli. After induction (isopropyl-1-thio-β-D-galacto-pyranoside, 30 μM at 16–19 °C for 6 h) the recombinant fusion proteins were isolated from the bacterial lysates. After sonication in buffer A (containing 20 mM Tris/HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 5 mM dithiothreitol, 200 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride and 10 μM mg of leupeptin), the protein was purified from the soluble fraction by glutathione-agarose chromatography. Untagged PI4Kβ was produced by expression in the recombinant E. coli BL21 strain of E. coli. It was purified by affinity chromatography on a glutathione-agarose column.

**Assay of PI 4-kinase**—The activity of PI 4-kinase was measured as incorporation of radioactivity from [γ-32P]ATP into organic solvotent-extractable material (22). The standard reaction mixture for PI 4-kinase (50 μl final volume) contained 50 mM Tris/HCl, pH 7.5, 20 mM MgCl2, 1 mM EDTA, 1 mM PI-4% Triton X-100, 0.5 mg/ml bovine serum albumin, 100 μM [γ-32P]ATP, and the enzyme. All assay components except [γ-32P]ATP were preincubated with or without inhibitors for 10 min at room temperature. Reactions were started by the addition of [γ-32P]ATP and terminated after 10 min by the addition of 3 ml of CHCl3/CH3OH/37% HCl (200:100:0.75 (v/v/v)). The organic solvent phase was separated from the aqueous phase by adding 0.6 ml of 140 kDa, which was mixing vigorously, and standing for phase separation. The upper (aqueous) phase was discarded, and 1.5 ml of CHCl3/CH3OH/0.6 N HCl (3:48:47 (v/v/v)) was added to the lower phase, followed by mixing and phase separation. The lower phase was then transferred to scintillation vials, and, after evaporation, the radioactivity was measured in a liquid scintillation counter.

To test the effect of prephosphorylation of PI4Kβ on its lipid kinase activity, the enzyme immobilized on glutathione-Sepharose 4B was incubated with 50 mM Tris/HCl, pH 7.4, 1 mM MnCl2 in the absence or presence of 300 μM ATP for selected periods of time (0.5–5.0 h) at 37 °C. The reaction tubes were then transferred to ice and equally reconstituted with ATP, followed immediately by the addition of 4 mM TPEN (to chelate Mn2+). After washing one more time with 500 μl of 4 mM TPEN, a standard lipid kinase assay was performed (except that the unlabeled ATP concentration was 1 mM) for 10 min at room temperature. For studies on the effect of dephosphorylation, the enzyme was incubated in the presence of Mn2+ in the presence or absence of ATP and treated with TPEN as described above. After the last wash, the immobilized enzyme was washed with “dephosphorylation buffer” (50 mM Tris, pH 7.4, 150 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol) and aliquoted into four tubes for treatment with PP1 (4 units/100 μl) or PP2A (0.1 unit/100 μl) for 30 min at 25 °C. A lipid kinase assay was then performed on the proteins using 1 mM [γ-32P]ATP (2 μCi/tube).

**Analysis of Protein Kinase Activity**—The assay was either performed on the GST-fused enzyme immobilized on glutathione-Sepharose 4B or on the PreScission-cleaved protein. In some experiments, the endogenous or expressed PI4Kβ, immunoprecipitated from COS-7 cells (see below) was used in the kinase assays. The reaction was performed in 100 μl of 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 3–10 mM MnCl2, 40 μM ATP, and 5 μCi of [γ-32P]ATP for 30 min at 37 °C. After phosphorylation, the protein was subjected to SDS-PAGE, and phosphorylation was quantitated using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

In Vivo Labeling of PI4Kβ in COS-7 Cells—COS-7 cells were grown on 10-cm culture dishes to 80% confluence and transfected with 6 μg of plasmid DNA per dish using LipofectAMINE 2000 reagent (Life Technologies Inc.) according to the manufacturer’s instructions. Cells were either untransfected or transfected with PI4Kβ (16) or the pEGFP-N1 plasmid (CLONTECH, Palo Alto, CA) for control. The kinase-dead version of PI4Kβ (DE56A) was created by site-directed mutagenesis using the QuikChange kit from Stratagene (La Jolla, CA). The 94-base pair Mf6/Lux4I fragment containing the mutation was subcloned into the wild-type DNA, and the entire sequence of this insert was verified by dideoxy sequencing. Transfected cells were labeled with ortho-[32P]phosphate (0.1 μCi/ml) for 4 h in phosphate-free Dulbecco’s modified Eagle’s medium. Labeled cells were washed three times with 10 ml of ice-cold PBS before lysis in 1.3 ml of lysis buffer per plate (25 mM Tris/HCl, pH 8.0, 150 mM NaCl, 2.5 mM MgCl2, 2 mM EDTA, 40 mM β-glycerophosphate, 1% Nonidet P-40, 10 mM NaF, 10 mM sodium pyrophosphate, 0.5 mM Na3VO4, 1 mM dithiothreitol, 0.1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 1 μM okadaic acid). Cell lysates were sonicated, and after centrifugation, 1 μg of anti-PI4Kβ antibody (Upstate Biotechnology Inc., Lake Placid, NY) was added to the supernatant and incubated at 4 °C overnight. Immunoprecipitated proteins were collected by adding Protein A-Sepharose (Amersham Pharmacia Biotech) and washed 3–5 times with 0.6 ml of buffer before SDS-PAGE analysis. When treated with WT, cells were incubated with 10 μM WT for 10 min, and the medium was changed before 32P labeling.

**RESULTS**

**Bacterial Expression of PI 4-kinase β**—Expression of the bovine PI4Kβ as a GST fusion protein was attempted in the BL21 strain of E. coli. Induction of protein expression by incubation with isopropyl-1-thio-β-D-galactopyranoside (20–100 μM) caused the appearance of a protein of ~140 kDa, which was present largely in the insoluble fraction (not shown). At mild inducing conditions (30 μM isopropyl-1-thio-β-D-galactopyranoside; 16–19 °C), about 50% of the protein was recovered in the soluble fraction. It is noteworthy that no soluble protein was obtained in the bacterial strain, DH5α. The soluble fraction was purified by affinity chromatography on a glutathione-agarose resin (Fig. 1A), with a usual yield of about 0.5 mg of purified protein from 1 liter of bacterial suspension. Since thrombin cleavage caused degradation of PI4Kβ, mostly yielding 48- and 60-kDa fragments (in addition to free GST), we also created a plasmid that contained the PreScission protease cleavage site instead of the thrombin site. This protease successfully released intact PI4Kβ from the GST fusion protein. The purified enzyme was more stable on the agarose beads than in solution and retained its lipid kinase activity over 10 days at 4 °C. However, its elution from the beads with glutathione or by proteolytic digestion became less efficient upon storage at 4 °C, and substantial amounts of the protein remained bound to the beads.

**Kinetic Parameters of Recombinant PI4Kβ**—Kinetic parameters of the recombinant PI4Kβ were determined to compare its properties with those of the purified activity isolated from bovine brain. The Kᵢ value of the recombinant enzyme for ATP was found to be 422 μM, and the Kᵢ value for adenosine was 635 μM, values that are comparable with those of the purified bovine protein (23). Moreover, the sensitivities of the lipid kinase activity of the enzyme to the PI 3-kinase inhibitors, WT and LY 294002, were identical to those previously described for the mammalian enzyme (23) (Fig. 2B). The specific activity of the purified enzyme (0.8–1.3 μmol/min/mg of protein) was somewhat lower, but comparable with that of the purified yeast Pik1 (4.3 μmol/min/mg) (24). We cannot rule out the possibility...
that some of the protein might have become inactive during the preparation or that some modification or co-factor is needed for the optimal function of the recombinant protein.

**PI4Kβ Undergoes Autophosphorylation**—To test whether PI4Kβ also possesses protein kinase activity, we evaluated its ability to undergo autophosphorylation. Fig. 1B shows that in the presence of [γ-32P]ATP (and without the lipid substrate, PI) the enzyme became phosphorylated, and this phosphorylation was blocked by preincubation with 10 mM WT. No autophosphorylation was detected in the presence of the lipid substrate, PI (not shown). Autophosphorylation was more pronounced in the presence of Mn2+ (compared with Mg2+) and a feature also described for autophosphorylation of PI 3-kinases (9, 10). Optimal Mn2+ concentration was found to be at 3–5 mM (not shown), and the pH optimum for autophosphorylation was around neutral pH, rapidly falling at higher pH values (not shown). The pH dependence of autophosphorylation closely correlated with the previously reported pH sensitivity of the enzyme’s lipid kinase activity (23). The sensitivities of protein kinase activities to the PI 3-kinase inhibitors, WT and LY 294002, were very similar to those of the enzyme’s lipid kinase activity. Each of the inhibitors reduced both activities with similar IC50 values (100 nM for WT and 5 μM for LY 294002) (Fig. 2, A and B).

We next examined whether PI4Kβ can transphosphorylate another molecule of PI4Kβ. For this, we prepared the enzyme both in its GST fusion form and in its cleaved form using the PreScission protease. Both enzymes were able to undergo autophosphorylation (the cleaved enzyme appeared to be more active in this respect), and when mixed together, both proteins became phosphorylated (Fig. 3, lanes 1, 2, and 5). However, when the GST fusion protein was pretreated with WT (which irreversibly inhibits the enzyme) and the inhibitor washed out, this enzyme showed no autophosphorylation (Fig. 3, lane 3) and could not serve as an acceptor when mixed with the still active cleaved enzyme (Fig. 3, lane 4). These results suggested that autophosphorylation was most likely to take place in a residue that is in close proximity to the ATP binding site (in the absence of PI) and is held there by intramolecular interactions.

**Effect of Phosphorylation on Lipid Kinase Activity**—To test the effect of protein phosphorylation on lipid kinase activity, the enzyme was preincubated with 300 μM ATP for selected periods of time in the presence of 1–3 mM Mn2+. Under these conditions, phosphorylation of the protein occurs over a period of 5 h (Fig. 4A). After phosphorylation, Mn2+ was removed by washing the enzyme twice with 4 mM TPEN on ice (for a total
of 15 min) followed by a lipid kinase assay in the presence of Mg\(^{2+}\) and high concentration (1 mM) of ATP. Since Mn\(^{2+}\) treatment itself decreased the catalytic activity of the enzyme, the activities of the phosphorylated enzyme (incubated with Mn\(^{2+}\) plus ATP) were compared with those only treated with Mn\(^{2+}\) (without ATP) at each time point. As shown in Fig. 4A, PI kinase activity decreased parallel to the autophosphorylation of the enzyme. To test whether phosphorylation indeed was responsible for the activity loss, we used protein phosphatases, PP1 and PP2A, to dephosphorylate the enzyme. As shown in Fig. 4B, both phosphatases were able to dephosphorylate the protein, (PP1 was more active in this regard), and the effect of PP2A\(_{a}\) was reversed by the addition of 1 mM okadaic acid (okadaic acid was not tested on PP1). Phosphatase treatment of the prephosphorylated enzyme increased the enzyme’s PI kinase activity, and this effect was prevented by pretreatment of the phosphatase with okadaic acid (Fig. 4B). Similar phosphatase treatment was without effect on the PI kinase activity of the control, unphosphorylated protein (Fig. 4B). These results suggested that the decreased lipid kinase activity is reversible and is indeed due to autophosphorylation of the protein.

**Fig. 3.** Phosphorylation is not due to transphosphorylation of PI4K\(\beta\). Purified recombinant PI4K\(\beta\) cleaved by PreScission protease (PI4K\(\beta\)) was incubated alone (lane 5) or together with GST-tagged PI4K\(\beta\) (GST-PI4K\(\beta\)) that was preincubated with (lanes 3 and 4) or without (lanes 1 and 2) WT (10 \(\mu\)M) for 10 min and washed to remove the inhibitor. A protein kinase assay was then performed with ([\(\gamma\]-\(\text{P}\)]ATP as described under “Experimental Procedures.” Phosphorylated proteins were separated by SDS-PAGE and analyzed by a PhosphorImager after staining with Coomassie Blue. While both GST-PI4K\(\beta\) and PI4K\(\beta\) were able to autophosphorylate (lanes 1 and 5, respectively), WT-inactivated GST-PI4K\(\beta\) (lane 3) was not phosphorylated by PI4K\(\beta\) (lane 4).

**Fig. 4.** The effect of autophosphorylation on lipid kinase activity of PI4K\(\beta\). A, purified GST-PI4K\(\beta\) was incubated in the presence or absence of 300 \(\mu\)M ATP with 3 mM Mn\(^{2+}\) for selected periods of time to allow autophosphorylation of the enzyme (see autoradiogram and open circles). After several washes with the Mn\(^{2+}\) chelator, TPEN (to remove Mn\(^{2+}\)), a PI kinase assay was performed with saturating concentration of ATP. The ATP-dependent loss of activity was calculated by comparing the activities of the enzyme preincubated with or without ATP at each time point (closed circles). Representative results are shown from three similar observations. B, the effect of protein phosphatase treatment on the lipid kinase activity of prephosphorylated PI4K\(\beta\). GST-PI4K\(\beta\) was prephosphorylated with unlabeled ATP (dark columns) or treated identically without ATP (open columns) for 30 min as described for A. Before the lipid kinase assay, proteins were incubated for 30 min (at 25 °C) with PP1 or PP2A\(_{a}\) or with PP2A\(_{a}\) treated with okadaic acid (1 \(\mu\)M). In parallel experiments, the effects of phosphatases were tested on enzymes autophosphorylated with [\(\gamma\]-\(\text{P}\)]ATP. Means \(\pm\) range of two experiments are shown each performed in duplicates.
tant of PI4Kβ and found that despite the lack of its protein and lipid kinase activities (Fig. 6, A and B), it still was found to be phosphorylated in COS-7 cells. The phosphorylation of the kinase-inactive protein was again slightly inhibited by pretreatment with WT but significantly less than the wild-type protein (Fig. 5, A and B).

We also examined whether the immunoprecipitated enzyme (apparently already phosphorylated) can undergo further phosphorylation in vitro. As shown in Fig. 6, both the endogenous and expressed PI4Kβ (but not its kinase-dead form) was able to autophosphorylate after immunoprecipitation from COS-7 cells. This in vitro phosphorylation was completely prevented by pretreatment of the immunoprecipitated enzyme with WT. Autophosphorylation activity closely correlated with the PI kinase activity of the immunoprecipitates (Fig. 6B). These results together indicated that although some autophosphorylation may occur in vivo, especially when PI4Kβ is overexpressed, the majority of the phosphorylation is due to the action of additional kinase(s) that act upon the protein in COS-7 cells.

**Autophosphorylation of PI4Kβ Occurs within the C-terminal ATP-binding Catalytic Domain**—We took advantage of the internal thrombin cleavage site(s) found in the kinase to determine which part of the molecule is autophosphorylated. First, the enzyme was phosphorylated with [γ-32P]ATP (with or without WT pretreatment) followed by digestion with thrombin for 12 h at 4 °C. After digestion, the soluble fraction was analyzed by SDS-PAGE followed by autoradiography. In parallel experiments, the enzyme was labeled with [3H]WT and digested identically. As shown in Fig. 7A, phosphorylation and [3H]WT labeling showed almost identical digestion patterns, with labeling of a 48-kDa main band (and weaker labeling of a 52-kDa larger fragment and 35-kDa smaller fragments, the latter probably being a product of further proteolysis by contaminating proteases). The WT-binding site has been mapped within the ATP-binding catalytic domain to the conserved lysine residue (Lys802) in PI 3-kinase α (21) and to the identical Lys799 on PI 3-kinase γ (12), a residue also present in type III 4-kinases (4, 25). Therefore, we hypothesized that the labeled fragments contain the C-terminal catalytic part of the protein. The larger 60-kDa, presumably N-terminal, fragment showed no specific

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**Fig. 5.** Phosphorylation of PI4Kβ in COS-7 cells. Nontransfected COS-7 cells or cells either transfected with the bovine PI4Kβ, its kinase-defective (KD) mutant (D656A), or pEGFP-N1 (as control) were labeled with ortho[32P]phosphate for 4 h before lysis and immunoprecipitation with an anti-PI4Kβ antibody (or normal rabbit serum (NRS)). Immunoprecipitated proteins were washed and subjected to SDS-PAGE and Western blot analyses. Phosphorylation of the protein was quantitated by a PhosphorImager. A, phosphorylation and Western blot analysis is shown from a representative of three similar experiments. B, quantification of the effects of WT pretreatment on the phosphorylation state of PI4Kβ from three separate experiments. Phosphorylation is expressed as a percentage of the respective untreated controls (means ± S.E.).

**Fig. 6.** Protein and lipid kinase activities of PI4Kβ immunoprecipitated from COS-7 cells. COS-7 cells were transfected with bovine PI4Kβ, its kinase-defective (KD) mutant (D656A), or pEGFP-N1, (as control) for 36 h. PI4Kβ was immunoprecipitated from the cell lysates with an anti-PI4Kβ antibody followed by PI kinase and protein kinase assays as described under “Experimental Procedures.” Autophosphorylation and Western blot analysis (A), and PI kinase activity (B) is shown from one of two similar observations. In these experiments, WT treatment (1 μM) was performed on the immunoprecipitated proteins for 10 min before the kinase assays.
Although the thrombin-cleaved enzyme loses its PI kinase activity, consistent with the need for the more N-terminally located unique lipid kinase domain, it retained its WT binding activity as judged by labeling of the smaller fragment with \([^3H]WT\). To test the possibility that the isolated catalytic domain retains its autophosphorylating activity, the enzyme was digested with thrombin prior to the protein kinase assay. Under these conditions, the 48-kDa fragment was still able to autophosphorylate (in a WT-sensitive manner) (Fig. 7B), confirming that this fragment contains the C-terminal catalytic core of the protein, which retains its active conformation even after thrombin digestion. (The WT-insensitive phosphorylation of the 80-kDa band co-migrating with the N-terminal fragment (F2 + GST) is an endogenous bacterial protein that copurifies with GST-PI4K\(\beta\).) C, schematic representation of GST-PI4K\(\beta\) showing the proteolytic cleavage sites and the location of the catalytic domain.

**DISCUSSION**

The present study demonstrates that PI4K\(\beta\), one of the two cloned type III PI 4-kinases, can be expressed in prokaryotes in a functional form. The recombinant protein shares all of the characteristics of the mammalian enzyme, including ATP affinity, WT binding, and inhibition by higher concentrations of PI 3-kinase inhibitors. We also found that the enzyme undergoes autophosphorylation, which, in turn, decreases its subsequent PI kinase activity. While we were unable to achieve stoichiometric phosphorylation of the enzyme as yet (probably due to the rapid inactivation of the protein with \(\mathrm{Mn}^{2+}\) and the possible presence of some nonfunctional protein), the fact that PI kinase activity is inversely related to autophosphorylation and that dephosphorylation restores lipid kinase activity suggests that, as far as the functional protein is concerned, autophosphorylation is likely to be stoichiometric. Further studies are under way to ensure a better phosphorylation stoichiometry that would allow determination of the autophosphorylation site.

We also found that autophosphorylation does not take place in the presence of the lipid substrate, PI, and that the enzyme is unable to trans-phosphorylate. These findings indicate that the internal phosphorylation site and the inositol lipid head group compete for the position close to the terminal phosphate of ATP, and another molecule of the kinase cannot access this catalytic site. Our finding that the truncated C-terminal catalytic domain of PI4K\(\beta\) is still capable of autophosphorylation indicates that the phosphorylation site resides within the catalytic core of the protein and does not require the presence of the more N-terminal domains. In contrast, the lipid kinase activity of such a truncated protein was completely lost, consistent with the need for the presence of the unique lipid kinase domain for lipid phosphorylation. In the recently published
Vps34p and PI 3-kinase subunit both phosphorylated by PI 3-kinase. PI kinases have also been reported; IRS-1 was found to be proteins remains to be elucidated. Additional protein substrates of in vivo autophosphorylation. Conserved Ser/Thr residues that might be candidates for autophosphorylation.

Whether protein kinase activity and autophosphorylation of PI 4-kinase β are relevant to its biological functions is not clear at present. Although we found that both endogenous and expressed PI4Kβ are phosphorylated in COS-7 cells, the contribution of autophosphorylation is relatively minor and mostly presents with the overexpressed enzyme. Since the presence of the lipid substrate inhibits autophosphorylation, the latter is more likely to happen when the protein is in excess relative to its substrate. However, the endogenous or expressed enzyme immunoprecipitated from COS-7 cells still was able to autophosphorylate, also suggesting that the enzyme is not heavily phosphorylated in vitro. While these data do not indicate prominent autophosphorylation in the bulk of the protein, they cannot rule out that autophosphorylation could become important in specific compartments of the cell, since this enzyme is likely to be involved in several aspects of cellular functions (4).

Importantly, our data also suggest that the enzyme is phosphorylated in mammalian cells by additional kinase(s), and the significance of this phosphorylation in the regulation of the protein is yet to be determined. In this regard, it is noteworthy that mutation of one of the conserved serine residues (S1045F) in the C-terminal sequence of Pik1, the yeast homologue of PI4Kβ, was found to be catalytically impaired, yielding a phenotype defective in membrane traffic and secretion (27).

The role of phosphorylation in the regulation of PI 3-kinases has not been well established in all cases, although autophosphorylation and protein kinase activity of certain PI 3-kinases have been proposed to be important for their function(s). In the case of PI 3-kinase δ, autophosphorylation has been shown to occur in vivo and results in decreased lipid kinase activity (10). Similarly, PI 3-kinase α can phosphorylate its p85 regulatory subunit both in vitro and in vivo, decreasing the lipid kinase activity of the complex (9). In contrast, autophosphorylation of Vps34p and PI 3-kinase γ was reported not to change the enzyme’s lipid kinase activity (11, 12), and the physiological role of the autophosphorylation in the regulation of these proteins remains to be elucidated. Additional protein substrates of PI kinases have also been reported; IRS-1 was found to be phosphorylated by PI 3-kinase α after stimulation with insulin (28) or interferon α (29). In the case of PI 3-kinase γ, evidence is accumulating to suggest that it has additional protein substrate(s). In a recent study, it was shown that protein, but not lipid, kinase activity of PI 3-kinase γ was necessary for its stimulatory effect on MAP kinase activation (13), suggesting that the protein kinase activity of the enzyme on some downstream component is important for the function of this protein. There was no indication in our studies that PI 4-kinase β would be able to act on protein substrates.

In summary, we have expressed a functional PI 4-kinase β in a bacterial expression system. This enzyme is indistinguishable from its mammalian counterpart in its catalytic properties and inhibitor sensitivities. Characterization of the recombinant protein revealed its autophosphorylation, which, in turn, inhibits the enzyme’s PI kinase activity. While PI4Kβ was also found phosphorylated in mammalian cells, apparently only a minor component of this phosphorylation is due to autophosphorylation. Further studies are needed to clarify whether phosphorylation plays a role in the control of the enzyme and cellular PI4-phosphate synthesis.

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