Autophosphorylation of Type I Phosphatidylinositol Phosphate Kinase Regulates Its Lipid Kinase Activity*

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Phosphatidylinositol phosphate kinases (PIPks) have important roles in the production of various phosphoinositides. For type I PIP5Ks (PIP5KI), a broad substrate specificity is known. They phosphorylate phosphatidylinositol 4-phosphate most effectively but also phosphorylate phosphatidylinositol (3,4)-phosphate (PI(3,4)P₂), and phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P₃), resulting in the production of phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P₂), phosphatidylinositol 3-phosphate, phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P₂), phosphatidylinositol (3,5)-bisphosphate (PI(3,5)P₂), and phosphatidylinositol (3,4,5)-trisphosphate. We show here that PIP5KIs have also protein kinase activities. When each isozyme of PIP5KI was immunoprecipitated protein kinase is involved. In addition, type II PIP5KIs expressed in Escherichia coli also retains this protein kinase activity, thus confirming that no co-immunoprecipitated protein kinase is involved. In addition, the autophosphorylation of PIP5KI is markedly enhanced by the addition of PI. No other phosphoinositides such as phosphatidylinositol phosphate, phosphatidylinositol bisphosphate, or phosphatidylinositol trisphosphate have such an effect. We also found that the PI-dependent autophosphorylation strongly suppresses the lipid kinase activity of PIP5KI. The lipid kinase activity of PIP5Kl was decreased to one-tenth upon PI-dependent autophosphorylation. All these results indicate that the lipid kinase activity of PIP5KI that acts predominantly for PI(4,5)P₂ synthesis is regulated by PI-dependent autophosphorylation in vivo.

The intracellular multifunctions of phosphoinositides (1–3) are regulated by a series of their metabolizing enzymes including lipases, kinases, and phosphatases (4–7). Phosphatidylinositol kinase and phosphatidylinositol phosphate kinase (PIPK) in particular are thought to be important for the spatiotemporal production of each phosphoinositide that directly controls a variety of functions.

The lipid kinases, which commit at the final step in the synthetic pathway of PI(4,5)P₂, PIPks have been identified and characterized. From their biochemical characteristics, they have been divided into two subtypes (type I and type II) (8). Primary sequences of type I and II PIPK revealed that these lipid kinases are conserved from yeast to mammal and form a family distinct from other lipid kinases (9–11). To date, three isoforms for each PIPK subtype (PIPKIα, -β, and -γ and PIPKIIα, -β, and -γ) in mammal have been identified (9–16). A FAB1 homolog in mammal (p235 PIKfyve) has also been reported recently (17). In data bases, sequences that seem to belong to this lipid kinase family are found also in fission yeast, nematode, and fruit fly, as well as in higher plants. A comparison of the primary sequences of types I and II PIPK revealed that these two subtypes are not so closely related (28–33%), whereas isoforms of the same subtype are highly homologous to each other (66–78%). In addition, type II PIPK shows relatively low enzymatic activity for PIP isolated from natural phospholipids as a substrate. A recent study has succeeded in explaining these differences between types I and II PIPks. Rameh et al. (18) showed that type II PIPK is a PI5P 4-kinase (PIP4K), and type I isofrom is exactly a PI4P 5-kinase (PIP5K). They also proved the existence of PI5P in vivo, a novel phosphoinositi-
phosphorylate only PI and thus produce only PI3P. Furthermore, PIP3Ks are known to have a Mn²⁺-dependent protein kinase activity. One class I PIP3K, P110α, phosphorylates p85α regulatory subunit at Ser-608 in the presence of Mn²⁺ (21, 22). In the case of p110γ and -δ, autophosphorylation of the catalytic subunit occurs most predominantly (23, 24). Upon treatment with wortmannin or a point mutation within p110γ which diminishes its lipid kinase activity, the protein kinase activity is also lost, suggesting the latter activity is based on almost the same mechanism as the former (22–24). Upon autophosphorylation, lipid kinase activities of p110α and p110β were strongly suppressed, indicating a mechanism for down-regulation through phosphorylation (21, 22, 24). A class III PIP3K, VPS34p, has also been shown to have a Mn²⁺-dependent protein kinase activity (25). This activity is also diminished in a lipid kinase-negative mutant. The lipid kinase activity of VPS34p is not affected by autophosphorylation (23, 25).

Evidence has been presented for the protein phosphorylation of the PIPK family. In platelets, PIP4KIIα has been shown to be phosphorylated, and a relationship between its phosphorylation state and lipid kinase activity has been suggested (26). It has also been shown that translocation of PIP4KIIα to the cytoskeletal fraction of platelets in response to thrombin is suppressed, indicating a mechanism for down-regulation through phosphorylation (21, 22, 24). A class III PIP3K, VPS34p, has also been shown to have a Mn²⁺-dependent protein kinase activity (25). This activity is also diminished in a lipid kinase-negative mutant. The lipid kinase activity of VPS34p is not affected by autophosphorylation (23, 25).

Here, we show that PIP5KI has a protein kinase activity. PIP5KI isoforms expressed both in COS-7 cells and in Escherichia coli autophosphorylate in vitro. The autophosphorylation level is enhanced specifically in the presence of PI. Lipid kinase activity of PIP5KI is strongly suppressed after the autophosphorylation in the presence of PI. These results suggest that the enzymatic activity of PIP5KI is regulated by its intrinsic protein kinase activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—PIP and PIP₂ were purified from bovine spinal cord as described (29) and were used as >99% pure PIP₄ and P1(4,5)P₂, respectively. PA, PI, PC, and PS were purchased from Doosan Serdary Research Laboratories. PIP₃ was purchased from Matreya, Inc. Synthetic PIP₅, P1(3,4)P₂, and P1(3,4,5)P₃ were generously donated by Dr. Watanabe (Ehime University). [γ⁻³²P]ATP was purchased from NEN Life Science Products. The polyvinylidene difluoride membranes used for Western blot analysis were from Nihon Eido. Ni²⁺-nitritotriacetic acid-garose was from Qiagen. The thin layer chromatography silica plates and separated by electrophoresis in pH 1.9 buffer (2% formic acid, 7.8% acetic acid) for the first dimension and pH 3.5 buffer (5% acetic acid, 0.5% pyridine) for the second dimension. The labeled phosphoamino acids were detected by autoradiography. The positions of the standard phosphoamino acids were detected by ninhydrin staining.

**Lipid Kinase Assay**—The lipid kinase reaction and detection of phosphorylated products were described previously (16). Briefly, PIPKs and substrate lipids (50 μM) were incubated in a kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM EGTA, 0.1 mM ATP, and 1 μCi of [γ⁻³²P]ATP) at room temperature for 20 min. The reaction was stopped by addition of 10 mM HCl and chloroform/methanol (2:1), phosphorylated lipids were separated by TLC and observed by autoradiography. (Alkaline Phosphatase Treatment—Myc-tagged form of P110α was immunoprecipitated from the lysate of overexpressing COS-7 cells. The immunoproteins were washed first with lysis buffer and then with alkaline phosphatase buffer (50 mM Tris-HCl (pH 8.2), 50 mM NaCl, 1 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), after which 2 units of calf intestine alkaline phosphatase (CIAP) (Takara Shuzo Co., Ltd.) or storage buffer for CIAP (10 mM Tris-HCl (pH 8.0), 1 mM MgCl₂, 50 mM KCl, 0.1 mM ZnCl₂, 50% glycerol) was added. The reaction was carried out at 30 °C for 60 min. To evaluate the effect of CIAP treatment on lipid kinase activity of Myc-PIP5KIα, the immunoproteins were washed five times with kinase buffer before lipid kinase assay.

**RESULTS**

**Type I PIP Kinases Autophosphorylate in Vitro**—To establish whether PIPK has any protein kinase activity, we carried out an in vitro kinase assay using each isoform of the PIPK members. Myc-tagged PIP5KIα, -β, and -γ or PIP4KIIα, -β, and -γ were transfected in COS-7 cells, respectively, immunoprecipitated with anti-Myc antibody, and incubated with [γ⁻³²P]ATP in the presence of Mg²⁺. All of the PIPK isoforms tested were phosphorylated, whereas no other phosphorylated protein was detected (Fig. 1). Furthermore, this phosphorylation was also observed when His-tagged PIP5KIα, -β, and -γ expressed in E. coli were used as enzyme sources (Fig. 2A), showing that no other co-immunoprecipitated protein kinase is involved. From these results, we concluded that type I isoforms of PIPK possess a protein kinase activity and autophosphorylate by themselves. On the other hand, we did not detect any phosphorylation when His-tagged type II PIP4Ks (PIP4KIIα, -β, and -γ) expressed in E. coli was used (data not shown). Thus we conclude that the phosphorylation of PIP5KIIα observed in Fig. 1 is due to some co-immunoprecipitated protein kinase present in amounts below the detectable limit. Thus, the autophosphorylation activity seems to be a unique characteristic of the type I PIPK isoform.

**The Protein Kinase Activity of Type I PIP Kinase Is Enhanced**
Specifically by PI—As the lipid kinase activity of PIP5KI is known to be activated by PA (30), we next examined the possibility that the protein kinase activity of PIP5KI is also modified by any phospholipid. The His-tagged form of PIP5KI was subjected to an in vitro kinase assay in the absence or presence of 50 μM PA, PC, PI, and PS, respectively. PI strongly stimulated the activity for autophosphorylation (Fig. 2A), whereas the other phospholipids including PA had no effect. Furthermore, this activation of autophosphorylation is highly specific to PI as phosphatidylinositol phosphates such as PI3P, PI4P, PI5P (not shown), PI(3,4)P2, PI(4,5)P2, and PI(3,4,5)P3 did not have such an effect (Fig. 2B). Finally, we found that the autophosphorylation was stimulated by PI in a dose-dependent manner, most effectively at 10 μM PI (Fig. 2C). These results show that PIP5KI has a PI-dependent protein kinase activity, a quite unique dependence on phospholipid different from that of any other protein kinase.

All Isoforms of PIP5KI Subtype Autophosphorylate in the Presence of PI—We next tried to determine whether the PI-dependent activation of autophosphorylation is a common characteristic of all PIP5KI isoforms. Myc-tagged forms of PIP5KIα, -β, and -γ were immunoprecipitated with anti-Myc antibody and then subjected to an in vitro kinase reaction in the presence or absence of 50 μM PI. As shown in Fig. 3, all type I isoforms were revealed to have PI dependence for their autophosphorylating activity. This result indicates that PI-dependent protein kinase activity is a specific characteristic common to all PIP5KI isoforms.

PI-dependent Autophosphorylation of Type I PIP Kinase Correlates with Its Lipid Kinase Activity—PI 3-kinases have also been reported to possess a protein kinase activity (21–24). In these cases, substitutions of the amino acids essential for the lipid kinase activity diminish the protein kinase activity as well. To test whether the catalytic residue of PIP5KI is also involved in the protein kinase activity, a lipid kinase-negative mutant (K138A) of PIP5KIα was tested. Lys-138 in PIP5KIα is a conserved amino acid corresponding to the Lys that binds the α-phosphate of ATP in protein kinases such as cAMP-dependent protein kinase. A substitution of this residue with Ala results in complete loss of lipid kinase activity (14).

GST fusion protein of wild type and the mutant (K138A) of PIP5KIα was expressed in E. coli and purified by glutathione-Sepharose and then subjected to in vitro kinase assay. GST-PIP5KIα (K138A) lost almost all its lipid kinase activity (both PI 5- and PIP 5-kinase) (Fig. 4A, and Ref. 14). At the same time, the lipid kinase-negative mutant also lost PI-dependent protein kinase activity (Fig. 4B). This indicates that the lipid
kinase and the protein kinase activity of PIP5KI are based on the same structural mechanism for catalysis.

Cation Dependence of the PI-dependent Autophosphorylation of PIP5KI—We next studied the divergent cation dependence of the PI-dependent autophosphorylation of PIP5KI. An in vitro kinase reaction was started in the presence of various cationic cations. As shown in Fig. 5A, PIP5KI preferred Mg<sup>2+</sup> most, but it also utilized Mn<sup>2+</sup> for autophosphorylation (Fig. 5A). Under the same conditions, lipid kinase activities were also measured. The PIP 5-kinase activity of PIP5KI for PI(4,5)P<sub>2</sub> production was exclusively dependent on Mg<sup>2+</sup>, whereas the PI 5-kinase activity for PI(5)P production was dependent on both Mg<sup>2+</sup> and Mn<sup>2+</sup> (Fig. 5B). The same cation dependence of PI 5-kinase and PI-dependent autophosphorylation may indicate that both activities are based on a similar catalytic mechanism.

PI-dependent Autophosphorylation Suppresses Both PI5- and PIP5-kinase Activity of Type I PIP Kinase—We further investigated the effect of PI-dependent autophosphorylation on lipid kinase activity of PIP5KI. His-PIP5KIβ, which was immobilized on beads by an immunoprecipitation with anti-penta-His antibody, was subjected to in vitro kinase reaction with or without PI/PIP. After the reaction, the excess of ATP and PI/PPIP was washed away, and lipid kinase activities (PI 5- and PIP 5-kinase activities) were measured. Fig. 6A shows that both lipid kinase activities were almost completely lost after autophosphorylation induced by PI. In contrast, the in vitro kinase reaction with ATP and PI/PIP, which failed to enhance the autophosphorylation of PIP5KI, resulted in no change in the lipid kinase activities (Fig. 6, A and B). This indicates that the marked decrease in lipid kinase activity is not due to any denaturation of PIP5KI enzymatic activity during the lipid kinase reaction. Finally, the time course experiment showed that there is a negative relationship between the lipid kinase activity and the degree of PI-dependent autophosphorylation (Fig. 6C). These results suggest that the PI-dependent autophosphorylation strongly down-regulates the lipid kinase activities of PIP5KI.

Autophosphorylation Occurs on Serine and Threonine Residues in Vitro and in Vivo—To characterize further the protein kinase activity of PIP5KI, we carried out a phosphoamino acid analysis using Myc-PIP5KIα. We examined whether PIP5KI is phosphorylated in vivo by a metabolic 32P labeling of COS-7 cells that were transfected with Myc-PIP5KIα. Myc-PIP5KIα was immunoprecipitated and subjected to SDS-polyacrylamide gel electrophoresis (Fig. 7A). The result showed that PIP5KIα is a phosphoprotein in vivo. Myc-PIP5KIβ and 1γ were also revealed to be phosphorylated in vivo by the same experiment (not shown). We also found treatment with alkaline phosphatase restored the lipid kinase activity of PIP5KIα (Fig. 7B). This together with the data in Fig. 6 suggest that a portion of PIP5KIα is phosphorylated and down-regulated in vivo through autophosphorylation. Next, we cut out the phosphorylated band in Fig. 7A and performed a phosphoamino acid analysis for Myc-PIP5KIα. Fig. 7C shows that the phosphorylation in vivo occurred mainly on serine residues. The same assay after PI-dependent autophosphorylation of Myc-PIP5KIα in vitro revealed that the phosphorylation occurred on serine and, to a lesser extent, on threonine residues but not on tyrosine residues (Fig. 7C). These results suggest that PIP5KI possesses a serine/threonine protein kinase activity in vitro, and its phosphorylation in vivo may be explained partly by the autophosphorylation of PIP5KI.

DISCUSSION

The PIP kinase family has been reported to have a broad substrate specificity in vitro. PIP5KI is able to phosphorylate the D-5 position on not only PI4P but also PI, PI3P, and PI(3,4)P<sub>2</sub> to produce PI5P, PI(3,4)P<sub>2</sub>, PI(3,5)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub> (19, 20). The broad substrate specificity of PIP5KI is similar to that of class I PI 3-kinases that also phosphorylate the D-3 position on PI, PI4P, PI5P, and PI(4,5)P<sub>2</sub>. Moreover, PI 3-kinase, including the class I subfamily, has been reported to possess a Mn<sup>2+</sup>-dependent protein kinase activity (21–24). Thus, we tried to elucidate whether PIP5KI also possesses protein kinase activity. As we have demonstrated in this study, type I PIP5KI transfectants to COS-7 cells or produced by the E. coli expression system was autophosphorylated in vitro, showing these findings expand the paradigm of dual-specific kinases capable of phosphorylating both protein and lipid.

In addition to the case with PI 3-kinase and PIP kinase in this study, there are some reports showing that the dual specificity toward protein and lipid substrates could be applicable to phosphatases as well. PTEN/MMAC1 is a putative tumor suppressor gene product homologous to protein tyrosine phosphatases such as CDC14, PTP-IV1, and CPTPH (31). Interestingly, it has been reported that PTEN/MMAC1 dephosphorylates the D-3 position of PI(3,4,5)P<sub>3</sub> (32) as well as tyrosine-phosphorylated protein. These results indicate a close evolutionary relationship between protein- and lipid-kinases/phosphatases. However, there is no evidence that other lipid kinases, such as PI 4-kinase or diacylglycerol kinase, have protein kinase activity. Unlike PI 3-kinase or PTEN/MMAC1, the PIP kinases are dissimilar to any known protein kinase in primary structure. This still does not rule out the possibility that the PIP kinases are related to some protein kinase family members. Future work may reveal a close relationship between PIP5K and other protein kinases in their tertiary structure.

Furthermore, we observed that the autophosphorylation of PIP5KI was stimulated strongly and specifically by PI. The
stimulation by PI was highly specific, and other polyphosphoinositides such as PIP, PIP2, and PIP3 did not have such an effect. Although the structural mechanism for PI-dependent autophosphorylation is unclear, the mechanism behind the down-regulation may be anticipated from the crystal structure of PIP4KIIβ reported by Hurley and co-workers (33). PIP4KIIβ forms a flattened surface for interaction with PI5P in the lipid bilayer, and certain positively charged amino acids seem to be involved in the interaction with the phosphate group of the substrate phospholipid. When PI-induced autophosphorylation occurs at serine/threonine adjacent to those positively charged residues, the interaction between PIP5KI and phosphoinositides is interrupted, down-regulating the lipid kinase reaction.

The protein kinase activity is only detected as an autophosphorylation of PIP5KI, and none of the protein substrates for this activity are currently known. By using His-PIP5KIβ, we did not observe any significant phosphorylation of protein substrates such as myelin basic protein or histone H1 (not shown).

Recently, it was reported that the protein kinase activity of p110γ PI3-kinase is involved in the activation of the mitogen-activated protein kinase pathway (34). This shows that the protein kinase activity of PI 3-kinase has roles for not only down-regulation of lipid kinase activity but also phosphorylation of any downstream target protein to transduce signals. Future work will answer the question about the existence of protein substrates for PIP5KI.

We have shown that PIP5KI is phosphorylated (Fig. 7A) and down-regulated (Fig. 7B) in vivo in the resting cells. This phosphorylation is possibly caused by endogenous PI. Therefore, some phosphatases may be activated in response to extracellular stimuli and then dephosphorylate PIP5KIs. Indeed, we
found that PIP5KI is dephosphorylated in response to lysophosphatidic acid, a typical agonist that induces inositol phospholipid turnover. Subsequently, PIP5KI activities are increased, resulting in the synthesis of PI(4,5)P₂. This down- and up-regulatory mechanism possibly functions in vivo.

In summary, we found that type I PIPKs have protein kinase activities and autophosphorylate in a PI-dependent manner, and this phosphorylation down-regulates the lipid kinase activity of type I PIPK. These results show the general physiological mechanism by which lipid kinase is regulated through protein phosphorylation. At the same time, our results also show a possible regulation of type I PIPK activity that plays critical roles in inositol lipid-signaling systems.

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