Regulation of Phosphatidylinositol 4-Phosphate 5-Kinase from *Schizosaccharomyces pombe* by Casein Kinase I*

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Phosphatidylinositol (4)P 5-kinase (PtdIns(4)P 5-kinase) catalyzes the last step in the synthesis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2). PtdIns(4,5)P2 is a precursor of diacylglycerol and inositol 1,4,5-trisphosphate and is also involved in regulation of actin cytoskeleton remodeling and membrane traffic. To satisfy such varied demands in several aspects of cell physiology, synthesis of PtdIns(4,5)P2 must be stringently regulated. In this paper we describe extraction, purification, and characterization of PtdIns(4)P 5-kinase from the plasma membranes of *Schizosaccharomyces pombe*. We also provide evidence that PtdIns(4)P 5-kinase is phosphorylated and inactivated by Cki1, the *S. pombe* homolog of casein kinase I. Phosphorylation by Cki1 *in vitro* decreases the activity of PtdIns(4)P 5-kinase. In addition, and most importantly, overexpression of Cki1 in *S. pombe* results in a reduced synthesis of PtdIns(4,5)P2 and in a lower activity of PtdIns(4)P 5-kinase associated with the plasma membrane. These results suggest that PtdIns(4)P 5-kinase is a target of Cki1 in *S. pombe* and that Cki1 is involved in regulation of PtdIns(4), 5P2 synthesis by phosphorylating and inactivating PtdIns(4)P 5-kinase.

Inositol-containing phospholipids (phosphatidylinositols) are found in all eukaryotes and constitute 2–8% of total cellular phospholipids. Phosphatidylinositol (PtdIns) is synthesized in the endoplasmic reticulum and accounts for more than 80% of the total phosphatidylinositols (1). Phosphatidylinositol 4-phosphate (PtdIns(4)P) and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) are synthesized from PtdIns by the sequential actions of PtdIns 4-kinase and PtdIns(4)P 5-kinase. In addition, PtdIns 3-kinase phosphorylates PtdIns(4,5)P2 and PtdIns at the 3 position of the inositol ring and adds to the complexity of PtdIns metabolism (1, 2).

PtdIns(4,5)P2 plays several fundamental roles in cell physiology. Phospholipase C catalyzes the hydrolysis of PtdIns(4,5)P2 to yield 1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate, two promiscuous diacylglycerol and inositol 1,4,5-trisphosphate, two promiscuous eukaryotic second messengers (3–8). PtdIns(4,5)P2 regulates actin microfilaments on the basis of its ability to bind to and regulate a host of actin severing, capping, and bundling proteins *in vitro* (9). PtdIns(4,5)P2 also binds to proteins with pleckstrin homology domains, such as pleckstrin or all phospholipase C isoforms (10, 11). In addition, PtdIns and its phosphorylated metabolites also play important roles in the regulation of membrane traffic (12).

Since the PtdIns(4,5)P2 metabolism plays such crucial roles in several aspects of cell physiology, it is logical that PtdIns(4,5)P2 synthesis and degradation are stringently regulated. Mammalian cells contain several isoforms of PtdIns(4)P 5-kinase with different biochemical properties. The type I PtdIns(4,5)P2 5-kinase is stimulated by both heparin and spermine. On the other hand, the type II PtdIns(4)P 5-kinase is inhibited by heparin, and inhibited or not affected by spermine (13, 14). In addition, the type I and not type II PtdIns(4)P 5-kinase is also stimulated by phosphatidic acid (15). This stimulation may be essential for resynthesis of PtdIns(4,5)P2 in response to PtdIns(4,5)P2 hydrolysis by phospholipase C and subsequent conversion of DG to phosphatidic acid. An additional mechanism for resynthesis of PtdIns(4,5)P2 may involve protein kinase C-mediated activation of the PtdIns 4-kinase and PtdIns(4)P 5-kinase. This speculation is based on the ability of phorbol esters and DG to elevate levels of PtdIns(4)P and PtdIns(4,5)P2 in intact cells (16). Consequently, elevation of DG levels following activation of phospholipase C may promote synthesis of PtdIns(4)P and PtdIns(4,5)P2. However, direct evidence for involvement of protein kinase C in activation of PtdIns 4-kinase and PtdIns(4)P 5-kinase is missing. On the other hand, small GTP -binding protein Rho was found to interact with and regulate PtdIns(4)P 5-kinase (17, 18). In addition, cAMP was implicated in regulation of PtdIns(4)P and PtdIns(4,5)P2 synthesis in *Saccharomyces cerevisiae* (19). However, Buxeda et al. (20) demonstrated that the 45- and 55-kDa forms of *S. cerevisiae* PtdIns 4-kinase are neither phosphorylated nor regulated by cAMP-dependent protein kinase.

Recently, type II (21, 22) and type I (23) PtdIns(4)P 5-kinases were cloned, and two *S. cerevisiae* genes, *MSS4* (24) and *FABI* (25), encoding PtdIns(4)P 5-kinase, were characterized. Zhang et al. (26) showed that type I PtdIns(4)P 5-kinases are able to utilize PtdIns(3)P to form PtdIns(3,4)P2 and further phosphorylate PtdIns(3,4)P2 to generate PtdIns(3,4,5)P3. This surprising finding identifies PtdIns(4)P 5-kinase as a key player capable of generating several polyphosphoinositide signaling molecules.

In this study we show that at least one form of PtdIns(4)P 5-kinase in *Schizosaccharomyces pombe* is associated with plasma membrane. We describe purification and characterization of this PtdIns(4)P 5-kinase and present evidence that this PtdIns(4)P 5-kinase is phosphorylated and regulated by casein kinase I (CK1) *in vitro* and *in vivo*.
Experimental Procedures

Materials—Protein purification from Plasma. Calibration proteins for electrophoresis were from Bio-Rad. Phospholipids were from Sigma and detersives from Pierce. [γ-32P]ATP was purchased from Amersham and [3H]inositol from American Radiolabeled Chemicals.

S. pombe and Media—S. pombe strain SP232 (h ade6-M216 leu1-2 ura4-D18) was grown at 32 °C in minimal medium (27) supplemented with 75 μg/liter of each of adenine, leucine, and uracil.

Purification of PtdIns(4)P 5-Kinase—Preparation of the membrane fraction was performed batchwise, starting from cells grown in 3 liters of YEA medium to A600 nm = 1.5. Cells were harvested by centrifugation (2,000 × g for 10 min), washed once with water and once with a phosphate buffer (20 mM potassium phosphate, pH 7.5, 1.4 M sorbitol, 10 mM NaCl, 0.5% 2-mercaptoethanol). The spheroplasts were prepared and lysed as described previously (28). The crude extract (∼150 ml) was centrifuged at 5,000 × g for 10 min, supernatant was removed and centrifuged at 25,000 × g for 30 min. The pellet (P2) from this spin was resuspended in 15 ml of sucrose solution (55% sucrose, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μM each of pepstatin, aprotinin, and leupetin). Aliquots of 2.5 ml were placed at the bottom of a 1.4 × 8-cm centrifuge tubes (each batch was processed in 6 tubes) and subjected to sucrose gradient fractionation as described previously (28, 29). Briefly, the pellet fraction in centrifuge tubes was overlaid with the following volumes and concentrations of sucrose (containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 20 μM each of pepstatin, aprotinin, and leupetin) at 0 °C: 1 ml ×50%, 1 ml ×45%, 1 ml ×40%, 1 ml ×35%, 1 ml ×30%, 1 ml ×25%, 1 ml ×20%, 1 ml ×15%, 1 ml ×10%, and 1 ml ×5%. The centrifugation (16 h ×170,000 × g, SW41 rotor), the gradients were fractionated from the top (600-μl fractions) and labeled sequentially as fractions 1–19. The pellet was resuspended in 600 μl of the sucrose solution and taken as fraction 20. All fractions in one tube were assayed in duplicate for marker enzymes, which included vanadate-sensitive marker enzymes for subcellular fractions were from Bio-Rad. Phospholipids were from Sigma and detersives from Pierce. [γ-32P]ATP was purchased from Amersham and [3H]inositol from American Radiolabeled Chemicals.

RESULTS

Phosphatidylinositol (4)P 5-Kinase Regulation by CK1

PtdIns(4)P 5-Kinase was subjected to phosphopeptide and phosphoamino acid analysis essentially as described (30, 31). After electrophoresis, the gel was exposed to film (Hyperfilm-MP, Amersham) at room temperature for several hours. The phosphorylated bands corresponding to PtdIns(4)P 5-kinase were excised, and assayed for radioactivity (28). Purified PtdIns(4)P 5-kinase (0.2 μg) was incubated with the indicated amounts of CK1 at 30 °C in 10 μl of the following buffer: 25 mM Mes, pH 6.5, 50 mM NaCl, 15 mM MgCl2, 2 mM EDTA, 100 μM ATP. Following the incubation with CK1, the reaction mixture was diluted to 100 μl with PtdIns(4)P 5-kinase assay buffer, and the activity of PtdIns(4)P 5-kinase was determined.

Phosphoamino Acid Analysis—The [32P]Labeled PtdIns(4)P 5-kinase was subjected to phosphopeptide and phosphoamino acid analysis essentially as described (30, 31). After electrophoresis, the gel was exposed to film (Hyperfilm-MP, Amersham) at room temperature for several hours. The phosphorylated bands corresponding to PtdIns(4)P 5-kinase were excised, and assayed for radioactivity (28). Purified PtdIns(4)P 5-kinase (0.2 μg) was incubated with the indicated amounts of CK1 at 30 °C in 10 μl of the following buffer: 25 mM Mes, pH 6.5, 50 mM NaCl, 15 mM MgCl2, 2 mM EDTA, 100 μM ATP. Following the incubation with CK1, the reaction mixture was diluted to 100 μl with PtdIns(4)P 5-kinase assay buffer, and the activity of PtdIns(4)P 5-kinase was determined.

Phosphopeptide Mapping—Cleavage of proteins by N-chlorosuccinimide was performed as described (32, 33). The [32P]Labeled PtdIns(4)P 5-kinase was subjected to SDS-PAGE and the gel was exposed to film at room temperature for 4 h. The bands corresponding to the phosphorylated PtdIns(4)P 5-kinase were excised and rinsed in 0.1 M (w/v) solution of urea in acetic acid and water (1:1). The gel slices were then incubated in 60 mM N-chlorosuccinimide in the above solution. After 3 washes in 10 mM Tris-HCl, pH 8.0, the gel slices were equilibrated in sample loading buffer before electrophoresis on 15% SDS-PAGE gel.

PtdIns(4)P 5-kinase Labeling of S. pombe Cells—The cells were grown in minimal medium to A600 nm = 1.5, harvested, and washed with isositol-free minimal medium, and cultured in 10 ml of the same medium containing 20 μM [3H]inositol (5 μCi/ml) for 16 h. The cells were then harvested, washed with water, resuspended in 2 ml of methanol, and disrupted with glass beads. The homogenate was acidified with 2 ml of 1 M HCl and extracted times with 2 ml of chloroform. The combined extracts were washed with 2 ml methanol, 1 M HCl (1:1) and concentrated under stream of nitrogen. TLC analysis was performed as described above and radioactivity corresponding to PtdIns, PtdIns(4)P, and PtdIns(4,5)P2 was determined by excising the appropriate piece of TLC plate and counting it in a liquid scintillation mixture.

Analytical Methods—Protein concentration was determined by the method of Bradford (34). Marker enzymes for subcellular fractions were assayed as described previously (28, 29).

RESULTS

PtdIns(4)P 5-Kinase Activity in Cell Lysates—The PtdIns(4)P 5-kinase activity is easily detectable in cell lysates of S. pombe,
However, the specific activity depends on the method of cell breakage. Osmotic lysis of spheroplasts consistently yielded about 2-fold higher specific activity of PtdIns(4)P 5-kinase (0.3 nmol/min/mg) than breakage with glass beads. Moreover, PtdIns(4)P 5-kinase activity was more stable in cell lysates prepared by the former method, probably because osmotic lysis of spheroplasts minimizes proteolysis. Therefore, we chose osmotic lysis of spheroplasts for preparation of cell lysates with PtdIns(4)P 5-kinase activity.

In pilot experiments to determine the subcellular distribution of *S. pombe* PtdIns(4)P 5-kinase, we found that the PtdIns(4)P 5-kinase activity was exclusively associated with particulate fractions, no activity was detectable in soluble fraction. When we performed differential centrifugation, almost 80% of the PtdIns(4)P 5-kinase activity was recovered in pellet fraction sedimenting between 5,000 × g and 25,000 × g (fraction P2). Approximately 10% of the activity was recovered in pellet fraction sedimenting between 25,000 × g and 170,000 × g (fraction P3).

**Subcellular Distribution of PtdIns(4)P 5-Kinase**—To determine the subcellular localization of PtdIns(4)P 5-kinase, the P2 pellet fraction was further purified on sucrose density gradient and assayed for organelle-specific enzyme markers (28) as well as for the PtdIns(4)P 5-kinase activity (Fig. 1). PtdIns(4)P 5-kinase cofractionated with vanadate-sensitive ATPase, which is a marker for the plasma membrane fraction. The peak fraction had 27- and 34-fold higher specific activities of the ATPase and PtdIns(4)P 5-kinase, respectively, than the crude lysate. PtdIns(4)P 5-kinase did not comigrate with marker enzymes for mitochondria (cytochrome c oxidase), endoplasmic reticulum (NADPH-cytochrome c reductase), Golgi apparatus (GDPase), or vacuole (α-mannosidase) (Fig. 1).

To further characterize the PtdIns(4)P 5-kinase activity, we investigated the interactions between the protein and the plasma membrane. The plasma membranes purified by sucrose density gradient were incubated with one of the following: 1 M NaCl, 1% Triton X-100, 0.2 M Na2CO3, or 1 M NH2OH. After the incubation, membranes were centrifuged (170,000 × g) and the supernatants and the pellets were assayed for PtdIns(4)P 5-kinase activity. The best extraction and the highest specific activity in the supernatant was achieved by 1 M NaCl. More than 90% of the PtdIns(4)P 5-kinase was recovered in the supernatant. Because most of the other proteins remained associated with the membrane, this simple extraction procedure resulted in a further 10-fold purification of the kinase. The solubiliza-

**FIG. 1.** The PtdIns(4)P 5-kinase co-migrates with the plasma membrane fraction. The P2 pellet was subjected to density gradient centrifugation as described under “Experimental Procedures.” After centrifugation, gradients were fractionated from top (fraction 1) to bottom (fraction 20) and assayed for: A, protein amount (mg/fraction) and refractive index; B, PtdIns(4)P 5-kinase (nmol/min/fraction) and vanadate-sensitive plasma membrane ATPase (μmol/min/fraction); C, GDPase (nmol/min/fraction) and α-mannosidase (μmol/min/fraction); D, cytochrome c oxidase (nmol/min/fraction) and NADPH-cytochrome c reductase (nmol/min/fraction). For the purification of PtdIns(4)P 5-kinase, total of 4 batches, each consisting of 6 tubes, were processed this way. Gradient profile of one typical gradient tube is shown for illustration.
tion with other reagents resulted in a significantly reduced recovery of PtdIns(4)P 5-kinase in the supernatant. However, in this experiment it is difficult to distinguish between the effect of dissociation of PtdIns(4)P 5-kinase from the membrane and the effect of the solubilizing reagents on the activity. Nevertheless, this result shows that PtdIns(4)P 5-kinase is a peripheral membrane protein and that the association with plasma membrane is mediated by ionic interactions.

**Purification of PtdIns(4)P 5-Kinase**—The salt extract of the plasma membrane fraction was further fractionated by hydrophobic-interaction chromatography on a column of phenyl-Sepharose (Fig. 2A). This chromatography yielded only modest 3-fold purification. However, when we omitted this step, the quality of resolution in subsequent purification steps suffered. The next purification step featured gel filtration on a Sephacryl S-100 column (Fig. 2B). PtdIns(4)P 5-kinase eluted as a sharp peak corresponding to molecular mass of 65 kDa. This step efficiently removes high molecular weight contaminants that are difficult to eliminate using other methods. It also removes sodium chloride remaining from the previous step. The final chromatographic step is an anion exchange chromatography over a Mono-Q column (Fig. 2C), and yields a homogenous enzyme preparation with a high specific activity (Table I).

To prove that the enzymatic activity resides in this single band, we performed a renaturation assay. The purified protein was analyzed by SDS-PAGE and transferred to a nitrocellulose membrane. The nitrocellulose strip was washed, cut into 19 sections, and each section assayed separately for PtdIns(4)P 5-kinase activity. The only detectable activity corresponded exactly to the position of the Coomassie-stained band and therefore the purified band contains PtdIns(4)P 5-kinase (Fig. 3).

**Kinetic Characterization of PtdIns(4)P 5-Kinase**—The kinetic characterization of the enzyme showed that PtdIns(4)P 5-kinase is activated by nonpolar detergents and some phospholipids. Variety of detergents (CHAPS, cholate, deoxycholate, digitonin, β-octylglucoside, Triton X-100, Zwittergent 3-14, and Lubrol PX) were tested for their effect on PtdIns(4)P 5-kinase activity. The highest stimulation of PtdIns(4)P 5-kinase activity was obtained in the presence of Triton X-100, the optimal concentration was found to be 2 mM, followed by an apparent inhibition of activity at concentrations above 2 mM. These results are indicative of surface dilution kinetics (35–37). Zwittergent 3-14 and Lubrol PX were less effective in stimulating PtdIns(4)P 5-kinase activity. Other tested detergents did not have any significant effect or inhibited PtdIns(4)P 5-kinase activity. These results suggest that PtdIns(4)P 5-kinase activity is stimulated only in the presence of detergents which have a long saturated hydrocarbon tail and are nonionic or zwitterionic.

We used the Triton X-100 detergent/phospholipid mixed micelle system to perform the kinetic characterization of the enzyme. The function of Triton X-100 in this system is to form a uniform mixed micelle with the substrate PtdIns(4)P. The Triton X-100 micelle serves as a catalytically inert matrix in which PtdIns(4)P is homogenously dispersed. In addition, the Triton X-100/PtdIns(4)P mixed micelle system allows an analysis of the enzyme in an environment that mimics the physiological surface of the membrane (38–42), where PtdIns(4)P 5-kinase functions. We determined dependence of the PtdIns(4)P 5-kinase activity on the surface PtdIns(4)P concentration using different set molar concentrations of PtdIns(4)P (Fig. 4A). In these experiments the molar concentration of...
PtdIns(4)P was kept constant, while the concentration of Triton X-100 was varied in order to produce the desired surface concentrations of PtdIns(4)P. PtdIns(4)P 5-kinase followed the surface dilution kinetics (35–37) since its activity was dependent on the surface concentration of PtdIns(4)P (Fig. 4A), and exhibited saturation kinetics with respect to the surface concentration of PtdIns(4)P. In addition, the dependence of PtdIns(4)P 5-kinase activity on PtdIns(4)P surface concentration was independent on the molar concentrations of PtdIns(4)P used in this experiment. The $V_{\text{max}}$ was determined to be 2.7 μmol/min/mg, and the $K_m$ value for PtdIns(4)P was 1.0 mol % (Fig. 4B). When PtdIns(4)P was kept at its saturating surface concentration (4.8 mol %; bulk concentration of PtdIns(4)P 0.1 mM, concentration of Triton X-100 2 mM), and the concentration of ATP was varied, the enzyme behaved according to the Michaelis-Menten kinetics with respect to the bulk concentration of ATP, and the $K_m$ for ATP was determined to be 8 μM.

Using the standard PtdIns(4)P 5-kinase assay, we determined the optimal pH for the PtdIns(4)P 5-kinase activity to be 7.5 (Fig. 5), and we tested several phospholipids and other compounds as possible effectors of PtdIns(4)P 5-kinase activity (Table II). Previously, spermine was shown to be a potent stimulator of type I PtdIns(4)P 5-kinase (14). Our results are consistent with this observation, 0.5 mM spermine increased the activity of the kinase to 300% (Table II). On the other hand, heparin at 30 μg/ml inhibited 50% of the activity of S. pombe PtdIns(4)P 5-kinase (Table II). Mammalian PtdIns(4)P 5-kinase, type I, was stimulated by heparin, whereas the type II was inhibited (14). On the basis of these results it appears that S. pombe PtdIns(4)P 5-kinase shares characteristics with both type I and type II mammalian PtdIns(4)P 5-kinases. Phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, and diacylglycerol did not significantly affect PtdIns(4)P 5-kinase activity, and PtdIns(4, 5)P$_2$ reduced the activity to 45% of the control. Phosphatidylinositol, phosphatidylserine (PS), and phosphatidic acid (PA) stimulated the kinase activity to 125, 160, and 185%, respectively. Since the regulation of PtdIns(4)P 5-kinase by PA and PS may be physiologically most significant, we performed a detailed kinetic analysis to explore the mechanism of phospholipid activation of PtdIns(4)P 5-kinase. The activity of PtdIns(4)P 5-kinase was determined at different surface concentrations of PtdIns(4)P at different set surface concentrations of PA or PS (Fig. 6). The addition of PA or PS to the assay system resulted in an increase of the appa-

**Table I**

<table>
<thead>
<tr>
<th>Process</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
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<tbody>
<tr>
<td>Crude extract</td>
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<td>3,154</td>
<td>0.32</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Pellet P2</td>
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<td>2,521</td>
<td>1.22</td>
<td>80</td>
<td>3.8</td>
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<td>Sucrose gradient</td>
<td>180</td>
<td>1,966</td>
<td>11</td>
<td>62</td>
<td>34</td>
</tr>
<tr>
<td>Salt extract</td>
<td>16</td>
<td>1,828</td>
<td>114</td>
<td>58</td>
<td>357</td>
</tr>
<tr>
<td>Phenyl-Sepharose</td>
<td>4.6</td>
<td>1,316</td>
<td>286</td>
<td>42</td>
<td>894</td>
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<tr>
<td>Sephacryl S-100</td>
<td>1.3</td>
<td>803</td>
<td>618</td>
<td>25</td>
<td>1,930</td>
</tr>
<tr>
<td>Mono Q</td>
<td>0.23</td>
<td>392</td>
<td>1,704</td>
<td>12</td>
<td>5,925</td>
</tr>
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</table>

**Fig. 3.** SDS-PAGE and renaturation of PtdIns(4)P 5-kinase. A, the purified protein was analyzed by 8% SDS-PAGE and transferred to a nitrocellulose membrane. The strip of nitrocellulose was washed, cut into 19 sections and each section was assayed separately for PtdIns(4)P 5-kinase activity (cpm). B, SDS-PAGE analysis of purified PtdIns(4)P 5-kinase. Lane 1, purified PtdIns(4)P 5-kinase (1.5 μg); lane 2, molecular weight standards.

**Fig. 4.** Dependence of PtdIns(4)P 5-kinase activity on the surface concentration of PtdIns(4)P. A, the PtdIns(4)P 5-kinase activity was measured as a function of the surface concentration of PtdIns(4)P (mol %). The molar concentration of PtdIns(4)P was held constant at 0.1, 0.075, or 0.125 mM, while the Triton X-100 concentration was varied. B, reciprocal plot of the data using PtdIns(4)P at a molar concentration of 0.1 mM. The line drawn is the result of a least-squares analysis of the data.
The phosphorylation of PtdIns(4)P 5-kinase by Cki1 was easily followed by phosphatidylglycerol assay. We examined the effects of GTP$\gamma$S on PtdIns(4)P 5-kinase activity. GTP$\gamma$S did not exhibit any effect on the kinase activity either when PtdIns(4)P was used as a substrate or when PtdIns(4)P 5-kinase was incubated with the following compounds for 20 min on ice and 5 min at 30 °C, and then assayed as described under "Experimental Procedures." The data represent the average of three assays which agreed within 5%.

### Table II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
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</thead>
<tbody>
<tr>
<td>No addition</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td>0.1</td>
<td>95</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>0.1</td>
<td>90</td>
</tr>
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<td>Phosphatidylycholine</td>
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<td>125</td>
</tr>
<tr>
<td>Phosphatic acid</td>
<td>0.1</td>
<td>185</td>
</tr>
<tr>
<td>Phosphatidylinositol (4,5)bisphtosamine</td>
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<td>45</td>
</tr>
<tr>
<td>Diacetylglcerol</td>
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<td>95</td>
</tr>
<tr>
<td>Inositol</td>
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<td>100</td>
</tr>
<tr>
<td>Spermine$^a$</td>
<td>0.5</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>230</td>
</tr>
<tr>
<td>Heparin</td>
<td>10 µg/ml</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>30 µg/ml</td>
<td>50</td>
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</table>

$^a$ Concentration of Mg$^{2+}$ was decreased to 2 mM in the assays with spermine addition.

The course of the phosphorylation by Cki1 (Fig. 8). While the full inactivation required transfer of 0.8 mol of phosphate per mol of PtdIns(4)P 5-kinase, the completely phosphorylated protein contained 3.5 mol of phosphate/mol of PtdIns(4)P 5-kinase. The phosphoamino acid analysis of PtdIns(4)P 5-kinase containing 0.8 mol of phosphate/mol of protein revealed that only serine was phosphorylated (Fig. 9). However, PtdIns(4)P 5-kinase containing 3.5 mol of phosphate/mol of protein contained, in addition to phosphoserine, also a detectable amount of phosphothreonine. Phosphotyrosine was not detected in either sample (Fig. 9).

To address a question whether phosphorylation of only one or several serine residues is responsible for inhibition of PtdIns(4)P 5-kinase, we cleaved the enzyme containing either 0.8 or 3.5 mol of phosphate/mol of protein with N-chlorosuccinimide. N-Chlorosuccinimide cleaves specifically tryptophanyl peptide bonds and generates discrete map of peptides (32, 33). Only a single phosphopeptide band of 18 kDa was detected in the sample with 0.8 mol of phosphate/mol of protein (Fig. 10, lane A). Since the maximum inactivation of PtdIns(4)P 5-kinase by Cki1 requires transfer of 0.8 mol of phosphate/mol of protein (Fig. 8), this result supports the interpretation that only a single serine residue is phosphorylated by Cki1 in the sample with 0.8 mol of phosphate/mol of protein and that phosphorylation of this serine residue regulates the activity of PtdIns(4)P 5-kinase. However, we cannot exclude the possibility that several serine residues within the 18-kDa N-chlorosuccinimide-resistant phosphopeptide are phosphorylated by
Cki1, and that the phosphorylation events synergistically in-activate PtdIns(4)P 5-kinase. Resolution of this issue will require a generation of specific PtdIns(4)P 5-kinase mutants by site-directed mutagenesis, when the sequence of \textit{S. pombe} PtdIns(4)P 5-kinase becomes available. In the sample with 3.5 mol of phosphate/mol of protein, in addition to the 18-kDa phosphopeptide, several additional phosphopeptide bands were detected (Fig. 10, lane B). Since phosphorylation beyond 0.8 mol of phosphate/mol of protein does not influence the activity of PtdIns(4)P 5-kinase.

We performed kinetic analysis to further characterize the effect of phosphorylation on the activity of PtdIns(4)P 5-kinase. Again, we measured the dependence of the PtdIns(4)P 5-kinase activity (phosphorylated to 0.8 or 3.5 mol of phosphate/mol of protein) on the surface concentration of PtdIns(4)P (Fig. 11). While the \(K_m\) of the phosphorylated enzyme for PtdIns(4)P did not change, the \(V_{\text{max}}\) was decreased to 1.0 \(\mu\)mol/min/mg. Also, the \(K_m\) value of the phosphorylated enzyme for ATP was not changed. We did not observe any difference in the above values between PtdIns(4)P 5-kinase containing 0.8 or 3.5 mol of phosphate/mol of protein.

To determine whether Cki1 regulates PtdIns(4)P 5-kinase activity also \textit{in vivo}, we measured PtdIns(4)P 5-kinase activity in the plasma membrane fraction purified from the wild type strain and the strain overexpressing Cki1 (Table III). Overexpression of Cki1 lowered the PtdIns(4)P 5-kinase activity to 48%. Incubation of purified plasma membrane fractions from the corresponding strains with recombinant purified Cki1 resulted in a further decrease of PtdIns(4)P 5-kinase activity. This effect was most pronounced with plasma membrane prepared from strain which did not overexpress Cki1. When wild-type \textit{S. pombe} cells and cells overexpressing Cki1 were labeled \textit{in vivo} with \(^{3}H\)inositol, and the total lipids were isolated and

**FIG. 8.** Inactivation of PtdIns(4)P 5-kinase by Cki1-mediated phosphorylation. PtdIns(4)P 5-kinase (0.2 \(\mu\)g) was phosphorylated with Cki1 (9 ng) for: A, 3 min or B, 20 min and subjected to SDS-PAGE. The bands corresponding to PtdIns(4)P 5-kinase were excised, the protein extracted and subjected to phosphoamino acid analysis as described under “Experimental Procedures.” The positions of the carrier standard phosphoamino acids are indicated.

**FIG. 9.** Phosphoamino acid analysis of the \(^{32}\)P-labeled PtdIns(4)P 5-kinase. PtdIns(4)P 5-kinase (0.2 \(\mu\)g) was phosphorylated with Cki1 (9 ng) for: A, 3 min or B, 20 min and subjected to SDS-PAGE. The bands corresponding to PtdIns(4)P 5-kinase were excised, rinsed, and treated with N-chlorosuccinimide as described under “Experimental Procedures.” The migration of the molecular mass standards (trypsin inhibitor, 22 kDa; lysozyme, 15 kDa) is indicated.

**FIG. 10.** Phosphopeptide mapping of the \(^{32}\)P-labeled PtdIns(4)P 5-kinase. PtdIns(4)P 5-kinase (0.4 \(\mu\)g) was phosphorylated with Cki1 (18 ng) for: A, 3 min or B, 20 min and subjected to SDS-PAGE. The bands corresponding to PtdIns(4)P 5-kinase were excised, rinsed, and treated with N-chlorosuccinimide as described under “Experimental Procedures.” The peptides were resolved on 15% SDS-PAGE and visualized by autoradiography. The migration of the molecular mass standards (trypsin inhibitor, 22 kDa; lysozyme, 15 kDa) is indicated.

**FIG. 11.** Effect of phosphorylation on the kinetics of PtdIns(4)P 5-kinase. PtdIns(4)P 5-kinase (0.2 \(\mu\)g) was phosphorylated with Cki1 (9 ng) for: A, 3 min or B, 20 min and subjected to SDS-PAGE. The bands corresponding to PtdIns(4)P 5-kinase were excised, rinsed, and treated with N-chlorosuccinimide as described under “Experimental Procedures.” The peptides were resolved on 15% SDS-PAGE and visualized by autoradiography. The migration of the molecular mass standards (trypsin inhibitor, 22 kDa; lysozyme, 15 kDa) is indicated.
fractionated by thin layer chromatography, we measured significant differences in the amounts of PtdIns(4,5)P$_2$ (Table III). The differences in PtdIns(4,5)P$_2$ synthesis were specific because the cellular contents of other phosphatidylinositols (PtdIns and PtdIns(4)P) were the same in both strains. The regulation of PtdIns(4,5)P$_2$ synthesis by Cki1 overexpression correlates well with the PtdIns(4)P 5-kinase activity measurements in the wild type and Cki1 overexpressing strains (Table III). These results suggest that PtdIns(4)P 5-kinase is a downstream effector of Cki1 in S. pombe, and that phosphorylation by Cki1 inactivates PtdIns(4)P 5-kinase.

**DISCUSSION**

The present paper describes the purification and characterization of PtdIns(4)P 5-kinase from plasma membranes of S. pombe and its regulation by CK1. The molecular mass of the S. pombe PtdIns(4)P 5-kinase (63 kDa) is similar to the 68-kDa type I PtdIns(4)P 5-kinase purified from bovine erythrocyte membranes (15) and 53-kDa type II PtdIns(4)P 5-kinase purified from cytosolic fraction of human erythrocytes. (13, 14). The $K_m$ values for PtdIns(4)P and ATP, stimulation by spermine and phosphatidic acid, and inhibition by heparin suggest that PtdIns(4)P 5-kinase purified from S. pombe shares characteristics with both type I and type II mammalian PtdIns(4)P 5-kinases.

Biochemical comparison with S. cerevisiae PtdIns(4)P 5-kinase homologs encoded by MSS4 (24) and FAB1 (25) genes is not possible, since both were identified solely on the basis of DNA sequence similarity with mammalian PtdIns(4)P 5-kinase (20). MSS4 encodes protein of 89 kDa, which upon overexpression suppresses mutations in PtdIns(4)P 5-kinase encoded by the STRT4 gene (24). FAB1 encodes protein of 257 kDa which is associated with the vacuole and is involved in regulation of vacuolar homeostasis. Neither MSS4 nor FAB1 were shown to be PtdIns(4)P 5-kinases by biochemical criteria. Our results do not suggest existence of additional PtdIns(4)P 5-kinase homologs in S. pombe. Cell fractionation and chromatographic separations always yielded only one peak of activity. However, we cannot exclude the possibility that another S. pombe homolog(s) with low activity escaped biochemical detection.

The presented data clearly show that Cki1 phosphorylates PtdIns(4)P 5-kinase in vitro and regulates its activity both in vitro and in vivo. In addition, the incorporation of $[^{3}P]$inositol in PtdIns(4,5)P$_2$ is regulated in a Cki1-dependent manner, and the overexpression of Cki1 results in a decreased activity of PtdIns(4)P 5-kinase associated with plasma membrane.

*S. pombe* contains four CK1 homologs encoded by cki1, cki2, hbp1, and hbp2. Cki1 (product of cki1 gene) associates with plasma membrane and its deletion or overexpression does not result in any observable phenotype (28). Like other members of the CK1 family (43–45), it is a constitutively active, monomeric protein kinase, and phosphorylates Ser and Thr residues downstream of Asp, Glu, or phosphorylated amino acid residues (28, 46, 47). Although many proteins, such as RNA polymerase I and II (48), p53 protein (49), regulatory subunit of protein phosphatase I (50), cytoskeletal proteins myosin, troponin, and ankyrin (50), the cAMP response element modulator CREM (51), and the 14-3-3 protein (52) were identified as substrates of CK1 in *in vitro*, only in a very few cases has the phosphorylation by CK1 been shown to correlate with functional changes of the substrate (53–57). As we demonstrate here, PtdIns(4)P 5-kinase is one of the few identified targets regulated by CK1 phosphorylation.

What is the significance of PtdIns(4)P 5-kinase regulation by CK1? Growing evidence obtained from a variety of experimental systems supports a direct role for inositol phospholipids in vesicular traffic events, distinct from their roles in classical signaling pathways (12). In this context it is important to note that recent results implicated CK1 of *S. cerevisiae* in vesicle transport (58, 59). YCK1, YCK2, and YCK3 (isoforms of CK1 in *S. cerevisiae*; Refs. 60 and 61) suppress in an increased dosage a growth defect of *gcs1* mutant cells. *GCS1* is a GTPase-activating protein (GAP) for monomeric GTP-binding protein ARF, which is involved in vesicle transport. The phosphorylation and inactivation of PtdIns(4)P 5-kinase by CK1 may explain the mechanism of this suppression. PtdIns(4,5)P$_2$ regulates ARF in a complex way: (i) it stimulates guanine nucleotide exchange factor and activates ARF by increasing the level of GTP-ARF (62), and (ii) PtdIns(4,5)P$_2$, together with phosphatidic acid, stimulates ARF GAP, which results in a lower level of GTP-ARF and lower activity of ARF (63). The *gcs1* mutation disrupts the GTP- GDP cycle on ARF and results in higher activity of ARF. The growth defect of *gcs1* cells was suppressed by overexpression of CK1, which inactivates PtdIns(4)P 5-kinase and lowers the level of PtdIns(4,5)P$_2$. This may result in reduced activities of ARF guanine nucleotide exchange factor and GTP-ARF, since PtdIns(4,5)P$_2$ is an activator of ARF guanine nucleotide exchange factor (62). The overexpression of CK1 may thus balance the activity of ARF and suppress the *gcs1* mutation. As shown by Poon et al. (64), imbalance in the GTP-ARF level in *S. cerevisiae* results in a severe growth inhibition. However, the involvement of CK1 in vesicle trafficking may be more complex. Panek et al. (59) isolated four mutations in *S. cerevisiae*, which eliminate the requirement for activity of YCK1 and YCK2. These mutations lie in four proteins with similarity to the four subunits of clathrin adaptors. Since clathrin adaptor proteins bind PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$ in mammalian cells, these results do not exclude the possibility that CK1 regulates vesicle

**Table III**

<table>
<thead>
<tr>
<th>Strain</th>
<th>In <em>in vivo</em> synthesis of inositol phospholipids</th>
<th>Specific activity of PtdIns(4)P 5-kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PtdIns</td>
<td>PtdIns(4)P</td>
</tr>
<tr>
<td>Wild type</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cki1 overexpression</td>
<td>106</td>
<td>95</td>
</tr>
</tbody>
</table>

* a Cells were labeled with $[^{3}P]$inositol, total lipids were extracted and separated by TLC, and the radioactive spots corresponding to individual phosphatidylinositols (PtdIns, PtdIns(4)P, and PtdIns(4,5)P$_2$) were measured by scintillation counting. The amounts of individual phosphatidylinositols in wild type *S. pombe* were taken as 100%.

b Plasma membranes were purified as described under "Experimental Procedures" and the peak fraction was used as a source of PtdIns(4)P 5-kinase by biochemical criteria. Our results do not result in any observable phenotype (28).

* Cki1 was overexpressed from nmt promoter of pREP1 plasmid as described previously (28).
trafficking at the plasma membrane by modulating the synthesis of PtdIns(4,5)P$_2$. It will be of interest to determine whether overexpression or depletion of YCK1 is reflected by changes in the activity of PtdIns(4)P 5-kinase or YCK2. It will be of interest to determine whether trafficking at the plasma membrane by modulating the synthesis of PtdIns(4,5)P$_2$. It will be of interest to determine whether overexpression or depletion of YCK1 or YCK2 in S. cerevisiae is reflected by changes in the activity of PtdIns(4)P 5-kinase associated with the plasma membrane and by synthesis of PtdIns(4,5)P$_2$.

REFERENCES
