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A novel phosphatidylinositol(3,4,5)P₃ pathway in fission yeast

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Article

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The mammalian tumor suppressor, phosphatase and tensin homologue deleted on chromosome 10 (PTEN), inhibits cell growth and survival by dephosphorylating phosphatidylinositol-(3,4,5)-trisphosphate (PI[3,4,5]P₃). We have found a homologue of PTEN in the fission yeast, Schizosaccharomyces pombe (ptn1). This was an unexpected finding because yeast (S. pombe and Saccharomyces cerevisiae) lack the class I phosphoinositide 3-kinases that generate PI(3,4,5)P₃ in higher eukaryotes. Indeed, PI(3,4,5)P₃ has not been detected in yeast. Surprisingly, upon deletion of ptn1 in S. pombe, PI(3,4,5)P₃ became detectable at levels comparable to those in mammalian cells, indicating that a pathway exists for synthesis of this lipid and that the S. pombe ptn1, like mammalian PTEN, suppresses PI(3,4,5)P₃ levels. By examining various mutants, we show that synthesis of PI(3,4,5)P₃ in S. pombe requires the class III phosphoinositide 3-kinase, vps34p, and the phosphatidylinositol-4-phosphate 5-kinase, its3p, but does not require the phosphatidylinositol-3-phosphate 5-kinase, fab1p. These studies suggest that a pathway for PI(3,4,5)P₃ synthesis downstream of a class III phosphoinositide 3-kinase evolved before the appearance of class I phosphoinositide 3-kinases.

Introduction

The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) was originally cloned as a tumor suppressor for gliomas (Li et al., 1997; Steck et al., 1997). We now know that PTEN is deleted or inactivated in many tumor types, including endometrial, breast, melanoma, and prostate (Simpson and Parsons, 2001). The PTEN protein is a phosphoinositide phosphatase specific for the D-3 position of the inositol ring (Maehama and Dixon, 1998). Although PTEN can dephosphorylate PI(3)P, PI(3,4)P₂ or PI(3,4,5)-trisphosphate (PI[3,4,5]P₃), it is likely that PI(3,4,5)P₃ is the main substrate in vivo. PI(3,4,5)P₃ synthesis occurs via phosphorylation of PI(4,5)P₂, a reaction that is catalyzed by class I PI 3-kinases (Hinchliffe and Irvine, 1997). PI(3,4,5)P₃ activates the Akt kinases, the Tec kinases, and several small G proteins, thereby stimulating cell motility, proliferation, and survival (Cantley, 2002).

Although classes I, II, and III PI 3-kinases are widely expressed in metazoan, only a single PI 3-kinase gene, vps34p, has been identified in yeast (Takegawa et al., 1995). Unlike the class I enzymes, vps34p synthesizes PI(3)P but not PI(3,4)P₂ or PI(3,4,5)P₃. PI(3)P is involved in the control of vesicle trafficking to the vacuole (Odorizzi et al., 2000). The failure to detect PI(3,4,5)P₃ (or PI(3,4)P₂) in yeast is consistent with the lack of a class I PI 3-kinase and has led to the assumption that no biosynthetic pathway for PI(3,4,5)P₃ exists in fission or budding yeast. Our observation that the Schizosaccharomyces pombe ptn1p has high homology to the mammalian PI(3,4,5)P₃ phosphatase, PTEN, led us to question this assumption. We find that ptn1p, like its mammalian orthologue, is a PI(3,4,5)P₃ phosphatase. Ptn1 disrupted (ptn1Δ) cells have levels of PI(3,4,5)P₃ comparable to mammalian

Abbreviations used in this paper: EMM, Edinburgh minimal medium; PI, phosphatidylinositol; PI(3,4,5)P₃, PI(3,4,5)-trisphosphate; PIP₅-kinase, PI-4-phosphate 5-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; ptn1Δ, ptn1 disrupted.
cells, display irregularly shaped vacuoles and are osmotically fragile. PI(3,4,5)P$_3$ synthesis in S. pombe required vps34p and its3p, but not fab1p. These results suggest a novel biosynthetic pathway for PI(3,4,5)P$_3$ that evolved before the appearance of class I PI 3-kinases.

Results

Identification of an S. pombe PTEN

Using a BLAST search, we identified an S. pombe gene (GenBank/EMBL/DDBL accession no. CAA22831) with significant homology to the mammalian PTEN. The putative phosphatase domain is 38% identical to the human PTEN phosphatase domain (Fig. 1 A). However, PTEN is closely related to dual specificity phosphatases that act on phosphoserine and phosphotyrosine, and the S. pombe gene might encode a dual specificity phosphatase. Based on the crystal structure of human PTEN (Lee et al., 1999), the residues required for PI(3,4,5)P$_3$ hydrolysis have been identified. Human PTEN has a 4-amino acid (amino acids 163–166) insert (relative to dual-specificity phosphatases) that increases the size of the active site. The S. pombe protein also has a 4-amino acid insert (Fig. 1 A, open box above sequence). Although the S. pombe insert is not similar to the human sequence, it places thr-167 and gln-171 (Fig. 1 A, hatched boxes) in frame with the corresponding human sequence. These two residues form hydrogen bonds with the PI(3,4,5)P$_3$ phosphates. The basic character of the human PTEN active site results from his-93, lys-125, and lys-128. Fig. 1 A (black boxes) shows that in the S. pombe sequence these residues are conserved. Finally, we examined the phosphatase signature sequence (also known as the P loop), which is a critical determinant of phosphatase specificity (Fig. 1 B). The human PTEN signature sequence is identical to mouse and Xenopus, and differs from Drosophila, C. elegans, and S. pombe proteins by a single isoleucine to valine substitution. In contrast, the Saccharomyces cerevisiae PTEN homologue is substantially different from human with four substitutions, consistent with reports that it does not hydrolyze phosphatidylinositol (PI) phosphates (Heymont et al., 2000; Maehama et al., 2001). Hence, the human and S. pombe genes are homologous, and the residues essential for PI(3,4,5)P$_3$ phosphatase activity are all conserved.

To test whether the S. pombe protein is a PTEN orthologue, we expressed the S. pombe gene product in bacteria and tested it for phosphoinositide phosphatase activity. The specific activity of the S. pombe putative phosphatase was similar to that of human PTEN. Both enzymes hydrolized PI(3,4,5)P$_3$, PI(3,5)P$_2$, and PI(3)P (Fig. 1 C). There was little or no activity toward PI(4,5)P$_3$. Based on the sequence homology and the phosphoinositide phosphatase activity, we conclude that this gene product is a functional orthologue of mammalian PTEN, which we designated ptn1p.

Ptn1p affects phosphoinositide levels in vivo

To test the role of ptn1 in vivo, we prepared a yeast strain (ptn1Δ) lacking ptn1 and then introduced a pREP1 ptn1p expression vector. These yeast strains were labeled with [3H]inositol, the lipids were extracted and deacylated, and the levels of phosphoinositide were analyzed by HPLC. The ptn1Δ cells had six- to eightfold increased levels of PI(3,4)P$_2$ and PI(3,4,5)P$_3$ as compared with wild-type cells (Fig. 2). Restoration of ptn1p levels with a ptn1p expression vector lowered PI(3,4)P$_2$ and PI(3,4,5)P$_3$ levels close to wild-type levels. Manipulation of ptn1p levels did not affect PI(3)P levels, indicating that in vivo PI(3)P is not a significant substrate for ptn1p, as has been suggested for human PTEN (Leslie and Downes, 2002). These results confirm that ptn1p is a PI(3,4,5)P$_3$ phosphatase in vivo and provide the first report of PI(3,4,5)P$_3$ in yeast.

Synthesis of PI(3,4)P$_2$ and PI(3,4,5)P$_3$ in S. pombe involves vps34p and its3p

In mammalian cells, the main pathway for PI(3,4,5)P$_3$ synthesis involves phosphorylation of PI(4,5)P$_2$ by a class I PI 3-kinase. Using BLAST searches of the S. pombe genome, we were not able to identify any putative class I PI 3-kinase genes, which are consistent with the experimental finding that vps34p is the only PI 3-kinase in yeast (Takegawa et al., 1995). To determine whether vps34p is critical for PI(3,4,5)P$_3$ production, we crossed a vps34Δ line (Takegawa et al., 1995) with ptn1Δ. The resulting line vps34Δ ptn1Δ had greatly reduced levels of PI(3,4)P$_2$ and PI(3,4,5)P$_3$ (Fig.

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Figure 1. Comparison of S. pombe PTEN proteins. (A) Alignment of human and S. pombe proteins (DNA Star program, J. Hein method). Open box, 4–amino acid insertion; shaded box, residues forming H-bonds with PI(3,4,5)P$_3$; black box, positive charges at active site. (B) Signature sequences for seven species. S. pombe protein is similar to PTEN in higher organisms. (C) Human and S. pombe PTEN phosphatases have similar specificities for PI substrates. Specific activities are reported as moles of phosphate released per mole of GST-PTEN per minute. Error bars are ± SEM.
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We also tested whether fab1p (Gary et al., 1998), a lipid kinase that converts PI(3)P to PI(3,5)P2 is involved in PI(3,4,5)P3 synthesis. The fab1Δ ptn1Δ line lacked PI(3,5)P2, but had PI(3,4)P2 and PI(3,4,5)P3 levels comparable to the ptn1Δ line (Fig. 3 B). These results demonstrate that vps34p, but not fab1p, is essential for production of PI(3,4)P2 and PI(3,4,5)P3 in S. pombe.

In mammalian cells, there is an alternative route for PI(3,4,5)P3 synthesis (Zhang et al., 1997; Tolias et al., 1998; Halstead et al., 2001). A PI-4-phosphate 5-kinase (PIP 5-kinase) mediates the conversion of PI(3)P to PI(3,4)P2 as well as the subsequent conversion to PI(3,4,5)P3. Its3p is the major PIP 5-kinase in S. pombe (Zhang et al., 2000) and, therefore, might play a role in the synthesis of PI(3,4,5)P3. Because disruption of its3 is lethal, we used the its3-1 line, which has ~10% of wild-type PIP 5-kinase activity (Zhang et al., 2000). We crossed the its3-1 cells with ptn1Δ cells and assayed phosphoinositide levels (Fig. 3 C). As expected, PI(4,5)P2 levels were decreased in its3-1 and its3-1 ptn1Δ cells. In addition, PI(3,4)P2 and PI(3,4,5)P3 levels were decreased in its3-1 ptn1Δ cells compared with ptn1Δ cells. The data are consistent with a model in which its3p, like its mammalian homologue, can convert PI(3)P to PI(3,4)P2 and PI(3,4,5)P3.

**Ptn1p affects vacuole morphology and osmotic fragility**

The ptn1Δ cells grew normally and had a normal morphology by bright field microscopy. However, using EM, we found that the ptn1Δ cells had misshapen vacuoles (Fig. 4). To quantify this phenotype, we counted the cells with at least 50% irregularly shaped vacuoles. Fig. 4 C shows that >70% of the ptn1Δ cells presented this phenotype. These findings demonstrate an effect of ptn1 disruption on vacuole morphology. We analyzed the ptn1p subcellular localization with a pREP42 GFP-pto1p expression vector. The GFP-pto1p fusion protein was detected in both punctate structures (0.5–1.0 μm in diameter) and septa of dividing cells (Fig. 5 A). As controls, we expressed a pREP42-GFP vector or an untagged pREP1-pto1p vector. We did not observe punctate or septal fluorescence.

We tested GFP-pto1p activity by osmotically stressing yeast cells (Fig. 5 B). In this assay (Paravicini et al., 1992), osmotic stress leads to cell lysis, release of alkaline phosphatase and formation of a bluish-green color. Inclusion of [3H]inositol into PI relative to PI phosphates varied considerably from sample to sample (1.0 ± 6.7, average ± SD). The inset shows a magnified view of the values for PI(3,4)P2 and PI(3,4,5)P3. (C) Fold increase in phosphoinositide levels as compared with the levels in wild-type cells. The data were normalized to give each wild-type phosphoinositide a value of 1.
of 1.2 M sorbitol inhibited color formation (unpublished data), indicating that cell lysis is, indeed, due to osmotic stress. In this assay, the ptn1Δ cells lysed more quickly than wild-type cells. Furthermore, expression of GFP-pto1p largely reversed this phenotype. These results demonstrate that GFP-pto1p is active and provide a simple, in vivo assay for pto1p activity. Osmotic fragility is usually associated with a cell wall defect, but further experiments are required to confirm this mechanism for the ptn1Δ cells.

Based on the lack of colocalization of GFP-pto1p with rhodamine phalloidin, the punctate structures were not associated with actin patches (unpublished data). To further characterize these punctate structures, we performed immuno-EM, using anti-GFP antibodies. Clusters of immunogold particles were detected in association with vesicular structures (Fig. 5 C, arrow). The gold particles were generally not associated with the larger vacuoles. Based on the size, we suspect that these structures may be endosomes (Prescianotto-Baschong and Riezman, 2002). Control cells that did not express GFP-pto1p did not show significant numbers of immunogold particles.

We next sought a PH domain protein that binds PI(3,4,5)P3 in vitro, although none showed high specificity for binding to this lipid compared with PI(4,5)P2. The first was a predicted protein designated SPAC 11E3.11C, which is a homologue of the ARNO/cytohesin/Grp family of Arf exchange factors (Fig. 6 A). The second was ksg1p, which is the S. pombe homologue of the mammalian PI(3,4,5)P3 regulated kinase, PDK1 (Niederberger and Schweingruber, 1999; unpublished data). Although the in vivo binding specificity of lipid binding domains often correlates with this in vitro assay, this is not always the case (Yu et al., 2004). A more reliable assay is relocation of the protein in vivo in response to a perturbation that alters phosphoinositide levels. In both wild-type and ptn1Δ cells, GFP-ksg1p showed septal and plasma membrane localization (unpublished data). A possible explanation is that the ksg1 PH domain targets the plasma membrane and septum via PI(4,5)P2 rather than PI(3,4,5)P3. In 83% (72/86) of ptn1Δ cells, the GFP-11E.11C protein localized to endosome-like structures, septa, and growing ends (Fig. 6 B), resembling the distribution of GFP-pto1p (Fig. 5 A). In contrast, examination of >100 wild-type cells showed no clear localization of the GFP-11E.11C (Fig. 6 C). These experiments establish 11E3.11C as a good candidate for a PI(3,4)P2/PI(3,4,5)P3–binding PH domain protein in S. pombe.

**Discussion**

Here, we have identified ptn1p, an S. pombe homologue of mammalian PTEN. The ptn1p phosphatase domain is 38% homologous to the human PTEN phosphatase domain, and
The observation that wild-type cells have undetectable or very low levels of PI(3,4)P2 and PI(3,4,5)P3 indicates that, as for mammalian cells, PI(3,4,5)P3 (and/or PI(3,4)P2) may function to recruit target proteins to specific subcellular locations via binding to protein modules. The \textit{S. pombe} genome includes 21 putative PH domains (Wood et al., 2002), which in mammalian cells bind to PI phosphates and mediate many of the downstream effects. Our investigation of the phosphoinositide binding specificity of \textit{S. pombe} PH domains revealed that the GFP-11E3.11C PH domain has distinct subcellular distributions in wild-type and \textit{ptn1}Δ cells, suggesting that it is regulated by PI(3,4)P2 and/or PI(3,4,5)P3. However, by the filter binding assay the 11E3.11C PH domain is not specific for PI(3,4)P2 or PI(3,4,5)P3. There are several possible explanations. First, there may be experimental complications, relating to incomplete folding of in vitro translated PH domains, thereby, compromising PH domain specificity. In addition, binding of PH domains to filters is an excellent method for surveying phosphoinositide specificity, but binding of PH domains to undiluted phosphoinositides on a filter is sometimes less selective than in biological membranes (Snyder et al., 2001). Second, specific binding of \textit{S. pombe} PH domains to membranes might require interactions with both lipid and protein targets. Indeed, some \textit{S. cerevisiae} PH domains require multiple interactions for membrane binding (Yu et al., 2004). Third, localization of the 11E3.11C PH domain in \textit{ptn1}Δ cells may be due to a higher affinity for PI(3,4,5)P3 than PI(4,5)P2, as has been observed for the ARNO PH domain (Venkateswarlu et al., 1998; Cullen and Chardin, 2000). The 11E3.11C PH domain is not specific for PI(3,4)P2 or PI(3,4,5)P3, suggesting that it is more selective than in biological membranes (Snyder et al., 2001).
50 mM Tris-HCl, pH 7.5, and 2 mM DTT for 30 min at 37°C. The reaction was stopped by addition of malachite green solution (BIOMOL Research Laboratories, Inc.), and the enzyme activity was measured by the change in absorption at 650 nm using appropriate controls.

**In vivo analysis of phosphoinositides**

Log phase cultures of yeast strains were grown in Edinburgh minimal medium (EMM) synthetic media plus appropriate supplements. Cells were washed twice in isoinositol-free EMM medium and subcultured (10^5 cells/ml) for 20 h in 5 ml of the same medium containing 10 µCi of myo-[2-3H]inositol. Labeled cells were harvested and lysed by vigorous vortexing with 0.5 ml 1 N HCl, 1 ml methanol-chloroform (1:1 vol/vol), and 1.5 g of acid-washed glass beads (Sigma-Aldrich). Bovine brain phosphoinositides (40 µg/sample; Sigma-Aldrich) were added as carrier lipid, and phase separation was induced by addition of 0.4 ml chloroform. The extracted lipids were decylated and analyzed by anion exchange high pressure liquid chromatography using a Partisphere SAX column (Agilent Technologies), using an online detector (Serunian et al., 1991).

**Microscopy**

For localization of GFP-ptn1, cells were grown to early log phase in EMM+adenine+leucine+thiamine, washed twice in EMM+adenine+leucine medium, and induced in the same medium for 24 h. For colocalization with actin, cells were fixed in 3.7% formaldehyde, stained with rhodamine phalloidin (Molecular Probes), and visualized with a Zeiss Axioskop and Apochromat 100X objective (n = 1.4). Micrographs were recorded with an AxioCam digital camera and OpenLab software (Improvision). For ultrastructural analysis, early log phase cells were fixed in 2% BSA 0.2% normal goat serum. Sections were stained with anti-GFP mAb (1:100; Covance) or a polyclonal rabbit antibody (1:50; Abcam). After washing, the sections were incubated with 10 nm of anti-antibody gold particles (Jackson ImmunoResearch Laboratories). The samples were washed and fixed after with 2% glutaraldehyde. The sections were stained with 1% uranyl acetate plus 1% lead citrate. Finally, the sections were briefly exposed to osmium vapor to provide additional contrast.

**Assay for osmotic integrity of yeast cells**

Yeast cells were overlaid with 0.05 M glycine HCl, pH 9.5, 1% agar, and 10 mM 5-bromo-4-chloro-3-indolyl phosphate (Paravicini et al., 1992). Assay for osmotic integrity of yeast cells

**References**


