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

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The Rockefeller University Press, 0021-9525/2004/07/205/7 $8.00

Key words: PTEN; phosphatidylinositol kinase; phosphatidylinositol-3-phosphate 5-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; ptn1 disrupted.

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 metazoa, only a single PI 3-kinase gene, vps34, 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

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The Journal of Cell Biology, Volume 166, Number 2, July 19, 2004 205–211
http://www.jcb.org/cgi/doi/10.1083/jcb.200404150

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cells, display irregularly shaped vacuoles and are osmotically fragile. PI(3,4,5)P₃ synthesis in ⁹ S. pombe required vps34p and its3p, but not fab1p. These results suggest a novel biosynthetic pathway for PI(3,4,5)P₃ that evolved before the appearance of class I PI 3-kinases.

Results

Identification of an ⁹ S. pombe PTEN

Using a BLAST search, we identified an ⁹ 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 ⁹ 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)P₃ 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 ⁹ S. pombe protein also has a 4–amino acid insert (Fig. 1 A, open box above sequence). Although the ⁹ 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)P₃ 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 ⁹ 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 ⁹ 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 ⁹ S. pombe genes are homologous, and the residues essential for PI(3,4,5)P₃ phosphatase activity are all conserved.

To test whether the ⁹ S. pombe protein is a PTEN orthologue, we expressed the ⁹ S. pombe gene product in bacteria and tested it for phosphoinositide phosphatase activity. The specificity of the ⁹ S. pombe putative phosphatase was similar to that of human PTEN. Both enzymes hydrolyzed PI(3,4,5)P₃, PI(3,5)P₃, and PI(3)P (Fig. 1 C). There was little or no activity toward PI(4,5)P₂. 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 [³²P]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(3,4,5)P₃ and PI(3,4,5)P₂, and PI(3,5)P₃ (Fig. 1 C). There was little or no activity toward PI(4,5)P₂. Based on the sequence homology and the phosphoinositide phosphatase activity, we conclude that this gene product is a functional orthologue of mammalian PTEN.

Synthesis of PI(3,4)P₂ and PI(3,4,5)P₃ in ⁹ S. pombe involves vps34p and its3p

In mammalian cells, the main pathway for PI(3,4,5)P₃ synthesis involves phosphorylation of PI(4,5)P₂ by a class I PI 3-kinase. Using BLAST searches of the ⁹ 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)P₃ 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)P₂ and PI(3,4,5)P₃ (Fig.
3 A). 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 ptn1Δ disruption on vacuole morphology. We analyzed the ptn1Δ subcellular localization with a pREP42 GFP-tpn1p expression vector. The GFP-tpn1p 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-tpn1p vector. We did not observe punctate or septal fluorescence.

We tested GFP-tpn1p 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 1.2 M sorbitol inhibited color formation (unpublished data), indicating that cell lysis is, indeed, due to osmotic stress. In this assay, the \textit{ptn1}/H9004 cells lysed more quickly than wild-type cells. Furthermore, expression of GFP-\textit{ptn1p} largely reversed this phenotype. These results demonstrate that GFP-\textit{ptn1p} is active and provide a simple, in vivo assay for \textit{ptn1p} activity. Osmotic fragility is usually associated with a cell wall defect, but further experiments are required to confirm this mechanism for the \textit{ptn1}/H9004 cells.

Based on the lack of colocalization of GFP-\textit{ptn1p} 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-\textit{ptn1p} did not show significant numbers of immunogold particles.

We next sought a PH domain protein that binds PI(3,4)P$_2$ and/or PI(3,4,5)P$_3$ and, thereby, mediates downstream signaling. The \textit{S. pombe} genome includes 21 proteins with predicted PH domains (Wood et al., 2002). Based on rules developed for mammalian PI(3,4,5)P$_3$-binding PH domains (Rameh et al., 1997; Lietzke et al., 2000), we identified seven candidates and tested them for phosphoinositide binding using filters spotted with lipids. Two of these PH domain proteins showed binding to PI(3,4,5)P$_3$ in vitro, although none showed high specificity for binding to this lipid compared with PI(4,5)P$_2$. 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 \textit{S. pombe} homologue of the mammalian PI(3,4,5)P$_3$ 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 relocalization of the protein in vivo in response to a perturbation that alters phosphoinositide levels. In both wild-type and \textit{ptn1}/H9004 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)P$_2$ rather than PI(3,4,5)P$_3$. In 83% (72/86) of \textit{ptn1}/H9004 cells, the GFP-11E.11C protein localized to endosome-like structures, septa, and growing ends (Fig. 6 B), resembling the distribution of GFP-\textit{ptn1p} (Fig. 5 A). In contrast, examination of >100 wild-type cells showed no clear localization of the GFP-11E.11C protein (Fig. 6 C). These experiments establish 11E.11C as a good candidate for a PI(3,4,5)P$_3$-binding PH domain protein in \textit{S. pombe}.

**Discussion**

Here, we have identified \textit{ptn1p}, an \textit{S. pombe} homologue of mammalian PTEN. The \textit{ptn1p} phosphatase domain is 38% homologous to the human PTEN phosphatase domain, and
all of the residues essential for PIP phosphatase activity are conserved. Furthermore, recombinant ptn1p dephosphorylates PI(3,4,5)P₃ and cells lacking ptn1 show markedly increased levels of PI(3,4)P₂ and PI(3,4,5)P₃. Based on these findings, we reach the surprising conclusion that S. pombe has a true PTEN orthologue that regulates the levels of PI(3,4)P₂ and PI(3,4,5)P₃.

The discovery of ptn1 led us to examine the biosynthetic pathway for PI(3,4,5)P₃ synthesis. We discovered a novel pathway that originates with synthesis of PI(3)P by vps34p, followed by the conversion of PI(3)P to PI(3,4,5)P₃. Its3p, the S. pombe orthologue of mammalian type I PIP 5-kinases, converts PI(3)P into PI(3,4)P₂, as has been shown to occur for mammalian type I PIP 5-kinases (Zhang et al., 1997; Tolià et al., 1998). The enzyme that catalyzes the last step in the synthesis of PI(3,4,5)P₃ has not been identified, but by analogy with the mammalian pathway, may also be its3p. 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, these lipids are tightly regulated in fission yeast. This regulation may occur at the level of synthesis and/or degradation of these lipids. Here we show that the ptn1p has an important role in maintaining the low levels of PI(3,4)P₂ and PI(3,4,5)P₃ in S. pombe. Understanding the spatial and temporal regulation of PI(3,4)P₂ and PI(3,4,5)P₃ synthesis are important questions for future studies.

Ptn1p, like PI(3)P (Gillooly et al., 2000) and vps34p (Stack et al., 1995), was found to localize with vesicular structures, and ptn1Δ cells show irregularly shaped vacuoles and are more readily lysed by osmotic stress. However, we also observed ptn1p associated with the septa of dividing cells. Hence, as in mammalian cells, PI(3,4,5)P₃ (and/or PI(3,4)P₂) in S. pombe likely has multiple functions, regulating different processes in different regions of the cell.

The mechanism by which this lipid affects cell function in fission yeast remains to be determined. One can imagine 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 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 S. pombe PH domains revealed that the GFP-11E3.11C PH domain has distinct subcellular distributions in wild-type and ptn1Δ 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 S. pombe PH domains to membranes might require interactions with both lipid and protein targets. Indeed, some S. cerevisiae PH domains require multiple interactions for membrane binding (Yu et al., 2004). Third, localization of the 11E3.11C PH domain in ptn1Δ cells may be due to a higher affinity for PI(3,4,5)P₃ than PI(3,4)P₂, as has been observed for the ARNO PH domain (Venkateswarlu et al., 1998; Cullen and Chardin, 2000). The 11E3.11C predicted protein is a homologue of the ARNO/cytohesin/Grp family and like these mammalian proteins, has an Arf GDP/GTP exchange domain and PH domain. Hence, PI(3,4,5)P₃ in lower eukaryotes may act through a PH domain (domains) that binds multiple phosphoinositides, and PI(3,4,5)P₃-specific PH domains may have evolved in more complex species.

In summary, the results presented here indicate that a pathway for the synthesis of PI(3,4,5)P₃ from PI(3)P existed in yeast before the evolution of class I PI 3-kinases in higher eukaryotes, indicating a more ancient function for this important signaling molecule.

Materials and methods

Generation of constructs, yeast strains, and recombinant proteins

The ptn1 ORF was amplified by PCR from S. pombe genomic DNA and subcloned into the pREP expression vector containing a thiamine repressible nmt-1 promoter. For localization studies, this PCR product was ligated to the 3’ end of a GFP cDNA in the pREP42 expression vector, which contains an attenuated version of nmt-1 promoter. To isolate recombinant proteins, the GST fusion construct of ptn1p was induced for 3 h in BL-21 cells, and the resulting fusion proteins were purified from bacterial lysates using glutathione Sepharose (Amersham Biosciences). All plasmids were verified by automated DNA sequencing. The ptn1Δ fission yeast strain was prepared by standard one step homologous recombination mediated gene replacement method. Stable integrants were selected in medium lacking uracil, and disruption of the gene was checked by PCR analysis. The vps34Δ (Takegawa et al., 1995), fab1Δ (Morishita et al., 2002), and its3-1 (Zhang et al., 2000) fission yeast strains were gifts from K. Takegawa (Kagawa University, Kagawa, Japan), C. Shimoda (Osaka City University, Osaka, Japan), and T. Kuno (Kobe University School of Medicine, Kobe, Japan). These strains were crossed with the ptn1Δ strain to generate appropriate double mutants.

Phosphatase assay

GST-PTEN and GST-ptn1 (1 μg/assay) were incubated with 25 nmols of appropriate dioctanoyl PI substrates in 500 μl of assay buffer, containing
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 isositol-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) were added as carrier lipid, and phase separation was induced by addition of 0.4 ml chloroform. The extracted lipids were deacylated 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 20 h. For colocalization system. The 35S-labeled proteins were incubated in 3% (wt/vol) fatty ethanol, embedded in Epon resin at 65°C overnight, and stained with lead citrate and uranyl acetate (Armstrong et al., 1993). Observation was based on examination of at least 100 cells. Digital images were prepared using a Partisphere SAX column (Agilent Technologies), using an online detector (Serunian et al., 1991).

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). The cells that lysed released alkaline phosphatase and turned bluish black.

Phosphoinositide binding by PH domains

Sequences containing the PH domains for ksg1 (residues 434-592), OBP1 (residues 254-350), OBP2 (residues 121-260), pob1 (residues 690-815), and SPBC 17G9.08C (residues 500-630) were in vitro transcribed and translated with [35S]methionine (Promega TNT coupled transcription/translation system). The [35S]-labeled proteins were incubated in 3% (wt/vol) fatty acid-free BSA (Sigma-Aldrich), 0.05% (vol/vol) fatty ethanol, 150 mM NaCl, 50 mM Tris, pH 7.5, with PVD membranes spotted with phospholipids. The synthetic diC6(1,2) phosphoinositides were from Cell Signals, Inc. and were spotted at 180, 60, and 20 pmoles per 100 nI spot. Phosphatidic acid was spotted at 180 and 60 pmoles. Ceramide, PI, sphingosine-1-phosphate were each spotted at 180 pmoles. After incubation with 35S-labeled proteins for 2 h at 4°C, the membranes were washed with 0.05% TWEEN 200, 150 mM NaCl, 50 mM Tris, pH 7.5. Proteins binding to lipid spots were detected by phosphorimaging.

Cailin Kennedy and Marie-Claire Daou provided excellent technical support. We thank Drs. Takegawa, Shimoda, and Kuno for S. pombe lines. Drs. Lambricht, Rhind, and Czech provided excellent advice. This research was funded by National Institutes of Health grants to L.E. Rameh, D. McComb, L.C. Cantley, and A.H. Ross.

Submitted: 26 April 2004
Accepted: 1 June 2004

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A yeast PTEN homologue


