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Characterization of *Saccharomyces cerevisiae* protein Ser/Thr phosphatase T1 and comparison to its mammalian homolog PP5

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Abstract

Background: Protein Ser/Thr phosphatase 5 (PP5) and its *Saccharomyces cerevisiae* homolog protein phosphatase T1 (Ppt1p) each contain an N-terminal domain consisting of several tetratricopeptide repeats (TPRs) and a C-terminal catalytic domain that is related to the catalytic subunits of protein phosphatases 1 and 2A, and calcineurin. Analysis of yeast Ppt1p could provide important clues to the function of PP5 and its homologs, however it has not yet been characterized at the biochemical or cellular level.

Results: The specific activity of recombinant Ppt1p toward the artificial substrates ^{32}P -myelin basic protein (MBP) and ^{32}P -casein was similar to that of PP5. Dephosphorylation of ^{32}P -MBP, but not ^{32}P -casein, was stimulated by unsaturated fatty acids and by arachidoyl coenzyme A. Limited proteolysis of Ppt1p removed the TPR domain and abrogated lipid stimulation. The remaining catalytic fragment exhibited a two-fold increase in activity toward ^{32}P -MBP, but not ^{32}P -casein. Removal of the C terminus increased Ppt1p activity toward both substrates two fold, but did not prevent further stimulation of activity toward ^{32}P -MBP by lipid treatment. Ppt1p was localized throughout the cell including the nucleus. Levels of *PPT1* mRNA and protein peaked in early log phase growth.

Conclusions: Many characteristics of Ppt1p are similar to those of PP5, including stimulation of phosphatase activity with some substrates by lipids, and peak expression during periods of rapid cell growth. Unlike PP5, however, proteolytic removal of the TPR domain or C-terminal truncation only modestly increased its activity. In addition, C-terminal truncation did not prevent further activation by lipid. This suggests that these regions play only a minor role in controlling its activity compared to PP5. Ppt1p is present in both the nucleus and cytoplasm, indicating that it may function in multiple compartments. The observation that Ppt1p is most highly expressed during early log phase growth suggests that this enzyme is involved in cell growth or its expression is controlled by metabolic or nutritional signals.

Background

Mammalian protein phosphatase 5 (PP5) and its homolog protein phosphatase T1 (Ppt1p) from the yeast *Saccharomyces cerevisiae* contain a catalytic domain structurally related to the catalytic subunits of PP1, PP2A and PP2B, and an N-terminal domain consisting of multiple tetratricopeptide repeats (TPRs) not found in other members of this family of phosphatases [1–3]. Homologs have also been identified in *Xenopus laevis* [4], *Neurospora crassa* [5], *Drosophila melanogaster* [6], *Trypanosoma brucei* [7], *Plasmodium falciparum* [8,9], and cauliflower [10], and homologs for *Caenorhabditis elegans*, *Schizosaccharomyces pombe* and *Arabidopsis thaliana* are predicted (accession number CAB60937, CAA17690 and AAD21727, respectively).

Protein phosphatase 5 has been implicated in the control of cell proliferation, in hormonal signal transduction, and in hsp90 chaperone function [3,11–17]. However little is known about the regulation of PP5 or its specific role in these pathways, and only one physiological substrate for PP5 has been identified to date, the apoptosis signaling kinase ASK 1 [13]. PP5 can be activated by lipid *in vitro*, and removal of the TPR domain or the C terminus generates an active, lipid insensitive form of the enzyme [18–21]. PP5 phosphatase activity is also altered by hCRY2 and a fragment of hsp90 *in vitro* [21,22]. These findings suggest that PP5 may be autoinhibited and that the binding of protein partners or lipids to the TPR domain could potentially control PP5 activity in cells, however no reports have yet documented how PP5 is regulated *in vivo*. The activity of PP5 homologs from cauliflower, *T. brucei* and *P. falciparum* is stimulated by polyunsaturated fatty acids *in vitro* [7–10] and removal of the TPR domain from *P. falciparum* PP5 increases activity to the same extent as lipid treatment [8,9]. This suggests that the TPR domain plays a similar inhibitory role for all PP5 homologs.

Genetic analyses in *S. cerevisiae* have yielded few clues about the function of Ppt1p. Budding yeast lacking *PPT1* do not exhibit obvious growth defects or detectable phenotypes [2]. Disruption of *PPT1* together with one of the other known phosphatases in budding yeast also reveals no obvious phenotype [23]. Thus, if Ppt1p has an essential function in *S. cerevisiae*, more than one other phosphatase can also carry out this role. In this report we have characterized the activity of recombinant Ppt1p, analyzed its expression at the level of protein and mRNA, and localized Ppt1p in yeast. Ppt1p can be activated by lipid, is most highly expressed in early log phase growth, and is present throughout the cell.

Results

Comparison of Ppt1p and PP5 activity and response to lipid treatment

Recombinant Ppt1p expressed as a GST-fusion protein, then affinity purified and released from GST by thrombin cleavage yielded a single band of the expected molecular weight, 58 kDa (Fig. 1A). When assayed side-by-side, the basal specific activities for recombinant Ppt1p and PP5 were similar toward the artificial substrates ³²P-MBP and ³²P-casein, respectively (Fig. 1B). Like PP5, the dephosphorylation of ³²P-MBP by Ppt1p was stimulated by arachidonic acid and other unsaturated fatty acids, but lipids lacking either a double bond or a carboxylate group were not effective (Table 1).

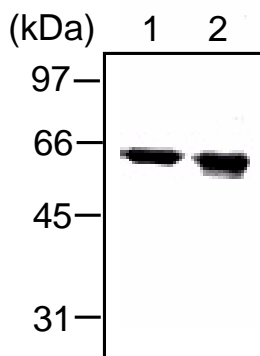
In the case of pNPP hydrolysis, others have reported both saturated and unsaturated fatty acyl coenzyme A esters stimulate PP5 activity, with concentrations required for half-maximal stimulation approximately 10-fold lower than those of their corresponding free fatty acids [21]. We therefore tested whether a saturated fatty acyl coenzyme A ester could also stimulate Ppt1p. Ppt1p activity toward ³²P-MBP was stimulated by arachidoyl coenzyme A in a concentration range similar to that observed for AA, with half-maximal stimulation occurring at approximately 50 μM for arachidoyl coenzyme A and 75 μM for AA. However, in contrast to AA stimulation, Ppt1p activity dropped off sharply at 80 μM arachidoyl coenzyme A and higher. Similar results were observed for PP5 (data not shown). This suggests that double bonds are not required to observe the effect of fatty acyl coenzyme A esters.

Unlike PP5, the activity of Ppt1p toward ³²P-casein was not stimulated by AA, arachidoyl coenzyme A, or linoleoyl coenzyme A over a range in concentration from 10–500 μM (Fig. 1B and data not shown). Other unsaturated fatty acids also failed to stimulate Ppt1p activity with this substrate (Table 1). These data indicate that Ppt1p is stimulated by polyunsaturated fatty acids, as well as by a saturated fatty acyl coenzyme A derivative, toward some but not all substrates dephosphorylated by PP5.

Limited proteolysis of Ppt1p

Removal of the TPR domain of PP5 by limited proteolysis increases enzyme activity and the TPR domain binds lipid [7–9,19,20]. Using conditions similar to those employed with PP5, digestion of Ppt1p with trypsin in the absence of lipid produced a 44 kDa catalytic fragment that was stable for at least 30 min and a 20 kDa fragment that was degraded within 30 min (Fig. 2A). N-terminal sequence analysis showed that these fragments are derived from the catalytic and the TPR domains, respectively (Fig. 2A,2B). The crystal structure of the PP5 TPR domain revealed three TPRs followed by an extended α helix [24], however Ppt1p was originally proposed to contain one additional

(A)



(B)

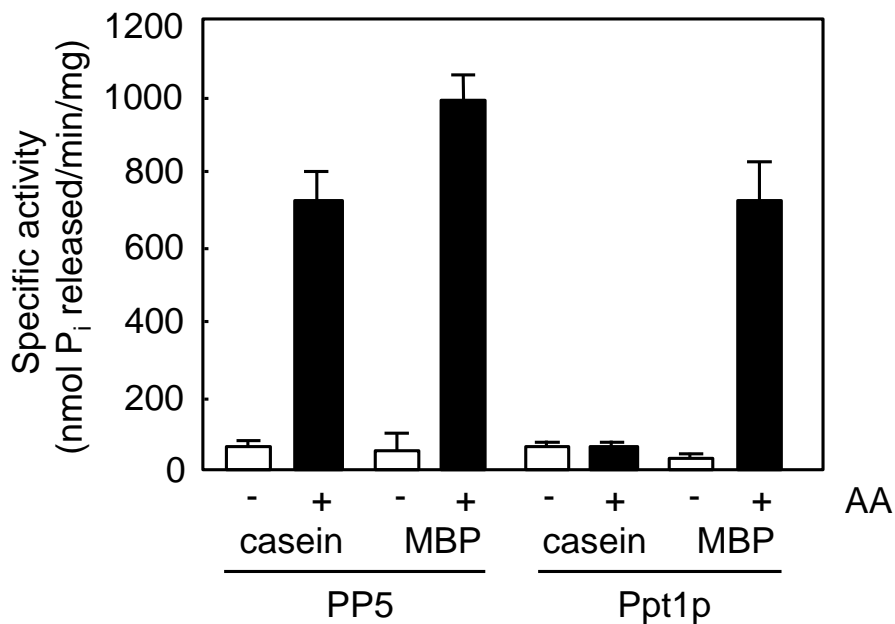


Figure 1

Comparison of the specific activities and response to lipid of Ppt1p and PP5. (A) Recombinant Ppt1p (lane 1) and Ppt1p (I-504) (lane 2) were expressed in *Escherichia coli*, purified by GST-agarose chromatography and cleaved with thrombin. Samples containing approximately 7.5 µg of protein were resolved by SDS-PAGE on a 10% polyacrylamide gel and visualized by Coomassie staining. The migration of molecular weight standards is indicated to the left. (B) Phosphatase activity of Ppt1p or PP5 was assayed for 10 min at 30°C in the absence (□) or presence (■) of 250 µM AA using 10 µM ³²P-MBP or ³²P-casein as substrate. Data are the average ± S.D. from assays performed in triplicate. Three independent experiments performed under similar conditions yielded similar results.

Table 1: Effect of various fatty acids on recombinant Ppt1p activity

Lipid added	Carbon chain	Relative Activity ^a		TPR protection
		Casein	MBP	
Arachidonic acid	20:4	1.3 ± 0.0	13 ± 0.5	+
Arachidic acid	20:0	0.6 ± 0.1	0.8 ± 0.3	-
Arachidonyl alcohol	20:4	0.7 ± 0.1	1.1 ± 0.1	-
Arachidonic methyl ester	20:4	0.7 ± 0.1	0.6 ± 0.2	-
Oleic acid	18:1	1.0 ± 0.1	16 ± 1.0	+
Linoleic acid	18:2	1.0 ± 0.1	21 ± 1.3	+
Palmitoleic acid	16:1	1.0 ± 0.1	20 ± 2.1	+

Phosphatase assays included Ppt1p that was incubated in the presence of various lipids each at a final concentration of 250 μ M. Assays also contained either 32 P-casein or 32 P-MBP. Data are presented as the average activity relative to control in the absence of lipid \pm S.D. from a single assay performed in triplicate. This experiment was repeated three times with similar results. The control values for recombinant Ppt1p were as follows: 79 ± 1.9 nmol Pi released $\text{minute}^{-1} \text{mg}^{-1}$ for 32 P-casein; 35 ± 10 nmol Pi released $\text{minute}^{-1} \text{mg}^{-1}$ for 32 P-MBP. A positive score for TPR protection indicates the sustained presence of multiple proteolytic fragments in the size range of 14 to 16 kDa after either trypsin or subtilisin digestion when compared to the control digest containing vehicle. ^aRatio of phosphatase activity in the presence of 250 μ M of the lipid indicated to the phosphatase activity without lipid.

TPR [2]. The pattern of proteolysis and sites of cleavage for Ppt1p are very similar to those seen with PP5 under these same conditions [19]. This indicates that Ppt1p also contains an accessible region linking the TPR and catalytic domains and is most consistent with the presence of three TPRs in Ppt1p. Limited tryptic proteolysis in the presence of 250 μ M AA generated a distinct pattern of fragmentation (Fig. 2A). A 20 kDa fragment was observed at early times, but a series of four fragments ranging from 14–16 kDa appeared between 10 and 30 min as the amount of the 20 kDa fragment decreased. These fragments, which are derived from the TPR domain, were not observed in the absence of AA, suggesting that lipid binding alters the conformation of this domain and its susceptibility to proteolysis. A similar change in susceptibility to proteolysis upon lipid binding was observed with PP5 [19]. As with PP5, only lipids that stimulated 32 P-MBP dephosphorylation slowed degradation of the 20 kDa TPR fragment (Table 1).

Removal of the TPR domain from Ppt1p by trypsin treatment had no effect on its activity toward 32 P-casein, but increased activity toward 32 P-MBP approximately two fold (Fig. 2C). AA did not stimulate 32 P-MBP dephosphorylation by Ppt1p after removal of the TPR domain (data not shown). Thus, lipids that activate Ppt1p alter the sensitivity of the TPR domain to proteolysis, and removal of the TPR domain causes a loss of the response of Ppt1p to lipid. However, removal of the TPR domain only modestly increases Ppt1p activity, in contrast to the 5–25 fold elevation reported after TPR removal from PP5 [19,20]. Although we cannot rule out the possibility that proteolyzed Ppt1p is unresponsive to lipid because it is unstable, the observations that the catalytic fragment remains

undegraded and control activity is not decreased argue against this possibility. Alternatively, an inhibitory element in the linker region may remain after TPR domain removal.

The effect of C-terminal truncation on Ppt1p activity

Removal of 10–13 residues from the C terminus of PP5 results in a highly active enzyme that is only modestly stimulated by lipid [19]. To examine the role of the C terminus in controlling Ppt1p phosphatase activity, a similar truncation mutant, Ppt1p (1–504), was expressed and characterized (Fig. 1A and 3). Compared to full-length enzyme Ppt1p (1–504) exhibited a two-fold increase in control specific activity with both 32 P-casein and 32 P-MBP. Lipid did not stimulate Ppt1p (1–504) toward 32 P-casein, but still increased activity with 32 P-MBP 12-fold. In contrast, C-terminal truncation of PP5 activates the enzyme as extensively as lipid treatment, and little further activation is seen with lipid [19]. In the case of PP5, subtilisin digestion removed both the TPR domain and the C terminus [19]. The observation that C-terminal truncation increased Ppt1p activity toward both 32 P-casein and 32 P-MBP, but subtilisin digestion increased activity only toward 32 P-MBP (see Fig. 2C) suggests that the C terminus of Ppt1p remained intact during subtilisin digestion. However, mass analysis of the subtilisin-derived catalytic fragment would be required to confirm this.

Expression and localization of HA-Ppt1p in yeast cells

To examine when Ppt1p is expressed and where it is localized, a yeast strain (WHT4-1) was generated in which the *PPT1* gene was replaced with cDNA encoding hemagglutinin-tagged Ppt1p (HA-Ppt1p). Immunoblotting showed that HA-Ppt1p levels were highest in early log phase, and

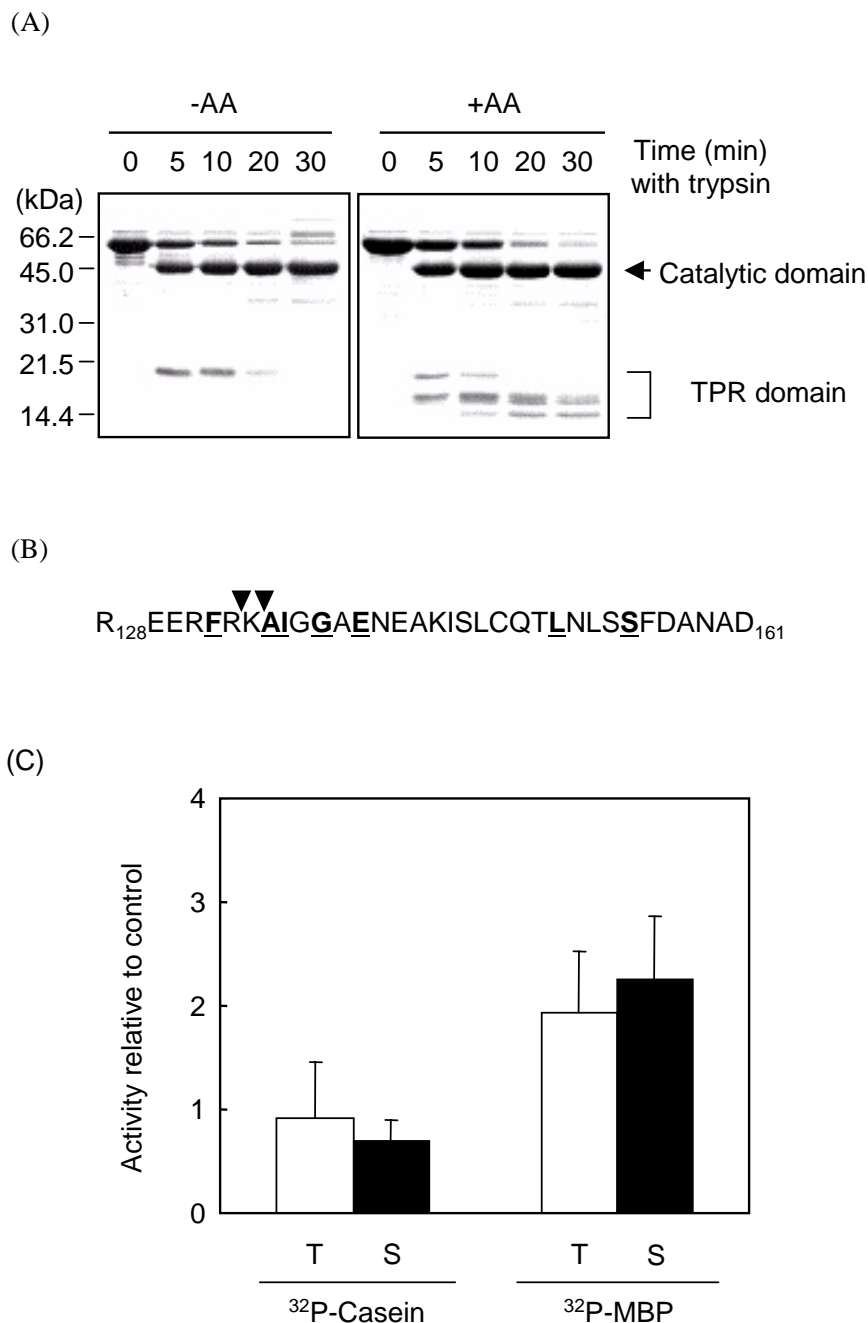
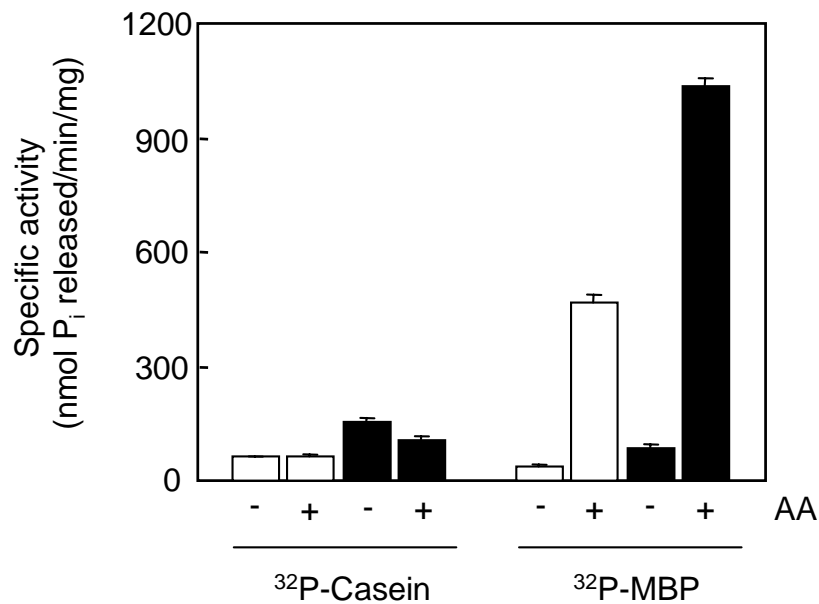


Figure 2
Limited proteolysis of PptIp and its effect on PptIp activity. (A) Recombinant PptIp samples trypsinized in the absence or presence of 250 μM AA were resolved by electrophoresis on 15% SDS-polyacrylamide gel and visualized by Coomassie staining. The length of digest (minutes) and the migration of molecular weight standards are indicated. (B) N-terminal sequence analysis of 45 kDa fragments shown in (A) was performed to identify the tryptic cleavage sites within the linker region between the TPR and catalytic domains. Tryptic cleavage sites are indicated with the inverted triangles. Bold underlined residues are conserved in both PptIp and PP5. (C) Recombinant PptIp was digested using either trypsin (T) or subtilisin (S) for 5 minutes in the absence of AA and assayed using either ³²P-casein or ³²P-MBP in the absence of AA. Data are presented as activity relative to control, which is the activity of full-length PptIp subjected to mock digest and assayed in the absence of AA. Results represent the mean ± S.E. from three or more independent experiments.

(A)



(B)

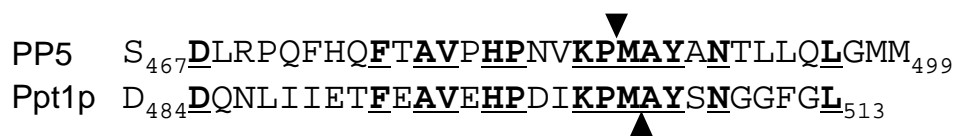


Figure 3

Effect of C-terminal truncation on PptIp activity. (A) Phosphatase activity of full-length PptIp (□) or the C-terminal truncation mutant, PptIp (1–504) (■), was determined in the absence or presence of 250 μM AA using ³²P-casein or ³²P-MBP as substrate. Data are the average ± S.D. of triplicate samples from a single experiment. Similar values were obtained in three independent experiments performed under the same conditions. (B) C-terminal sequences of PptIp and PP5 are aligned to display the truncation sites for PptIp (1–504) and PP5 (1–486) [19]. The truncation sites are indicated with the filled triangles. Bold underlined residues are conserved in both PptIp and PP5.

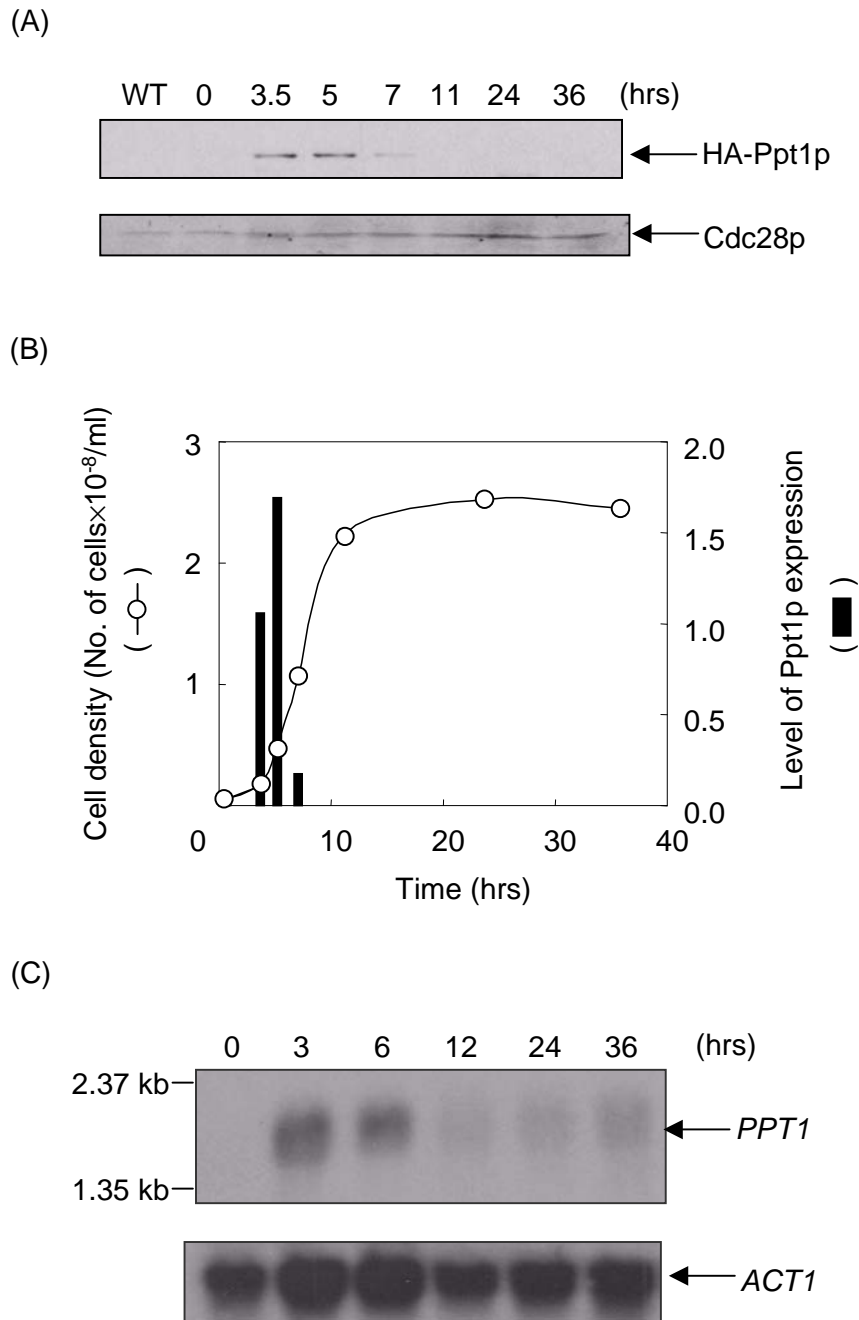
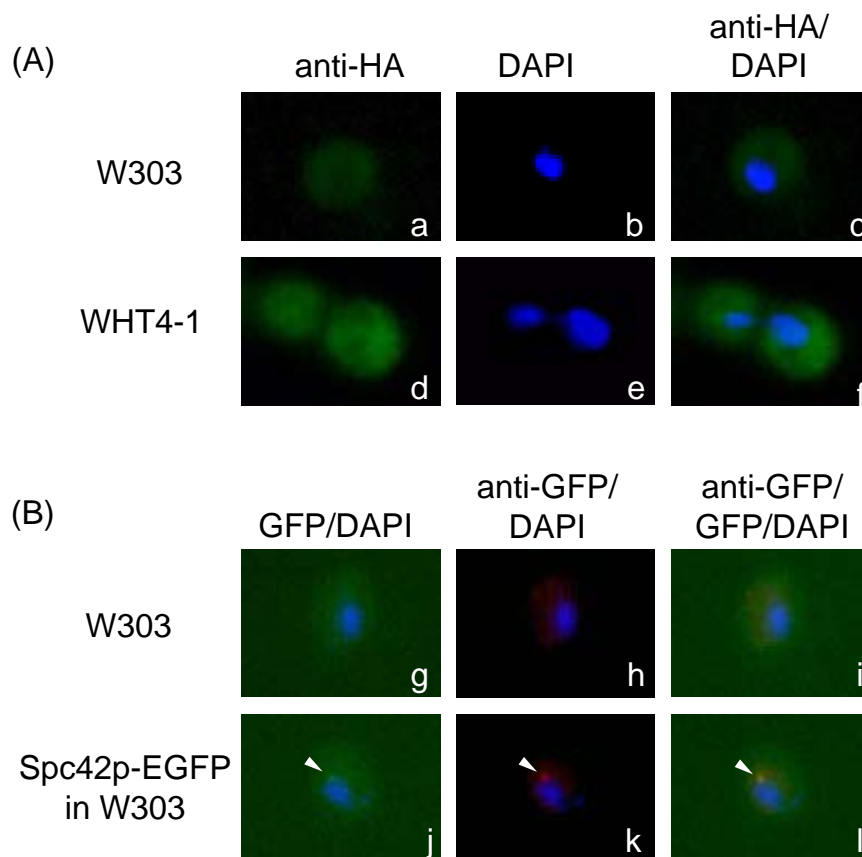


Figure 4

Expression of HA-Ppt1p as a function of cell growth. (A) An overnight culture of WHT4-I cells ($OD_{600\text{ nm}} 5\sim 10$) was used to inoculate fresh medium to $OD_{600\text{ nm}} 0.2$ and the culture grown at 30°C . At the indicated times samples were prepared and subjected to immunoblotting using anti-HA antibody. The lower panel shows the bottom half of the same blot probed with anti-Cdc28p antibody. This experiment was performed four times independently with similar results. WT; wild type. (B) The growth curve was plotted based on direct cell counting using a hemocytometer. The relative amount of HA-Ppt1p was obtained by taking the ratio of the band intensity of HA-Ppt1p to Cdc28p. (C) Total RNA was isolated at the indicated time after the inoculation of an overnight culture of W303 cells ($OD_{600\text{ nm}} 5\sim 10$) to $OD_{600\text{ nm}} 0.2$. Twenty micrograms of RNA per sample were resolved and subjected to Northern analysis with a ^{32}P -labeled *PPT1* cDNA probe. The lower panel shows the same blot probed for yeast actin mRNA. This experiment was repeated four independent times for both W303 and WHT4-I cells with similar results.

**Figure 5**

Localization of HA-Ppt1p in yeast cells during early log phase growth. (A) Yeast cells expressing wild-type Ppt1p (W303; a, b, c) or HA-Ppt1p (WHT4-1; d, e, f) were subjected to immunocytochemistry using modified ABC amplification. DAPI was used for nuclear staining. The exposure time for the HA-Ppt1p (green) signal was 5 sec. (B) A spindle pole body protein, Spc42p fused with EGFP (Spc42p-EGFP) was expressed in W303 cells, and localized by both direct green fluorescence of EGFP (j) and indirect red immunofluorescence (k) using the same method of amplification. The exposure times for the direct EGFP signal and the indirect immunofluorescence signal were 5 sec and 40 msec, respectively. Wild-type cells (W303) were used as negative controls for both experiments (a, b, c, and g, h, i). All samples were observed with 1000 × magnification.

then decreased dramatically during the stationary phase (Fig. 4A,4B). Northern analysis showed that the *PPT1* mRNA levels also increased in early log phase and decreased as the cell division decreased upon entering the stationary phase (Fig. 4C), correlating with protein expression. The pattern of *PPT1* gene expression was identical in wild-type cells and cells expressing HA-Ppt1p, indicating that the epitope tag did not alter transcription or message stability (data not shown).

Cells in early log phase growth when *PPT1* gene expression was maximal were used to localize HA-Ppt1p. Indirect immunofluorescence was required to detect HA-

Ppt1p. Fluorescence microscopy indicated that HA-Ppt1p was present throughout the cell, including the nucleus (Fig. 5A). Localization and staining intensity did not appear to change as a function of bud size or morphology, suggesting that Ppt1p expression and localization is not cell cycle dependent.

To confirm that the indirect immunofluorescence procedure did not generate artifacts, the localization of the spindle pole body protein Spc42p was also examined by this method. A yeast centromeric plasmid encoding the fusion protein Spc42p-EGFP was transformed into W303 cells. Direct fluorescence from EGFP and indirect

immunofluorescence using the amplification protocol with anti-EGFP antibody were compared. Both signals revealed specific localization of Spc42p-EGFP to the spindle pole body (Fig. 5B).

When HA-Ppt1p or EGFP-Ppt1p was overexpressed from the *GAL1* promoter in a centromeric or episomal plasmid, respectively, indirect immunofluorescence of HA-Ppt1p or direct fluorescence from EGFP-Ppt1p showed that both proteins were also localized throughout the cell.

Discussion

PP5 and its homologs in other species represent a sub-family of PPP enzymes distinguished by the presence of an N-terminal domain containing multiple TPR repeats. Ppt1p from the yeast *Saccharomyces cerevisiae* was the first homolog of mammalian PP5 to be described. Thus far, genetic approaches have not revealed functions for budding yeast Ppt1p. In this report, we have characterized Ppt1p activity *in vitro* and examined its expression.

In the case of mammalian PP5, results from lipid stimulation and binding, limited proteolysis and truncation studies taken together suggest the TPR domain and the C terminus inhibit PP5 activity in a coordinated manner and that lipid binding to the TPR domain activates the enzyme *in vitro* [18–20]. In the present study we used assay conditions for Ppt1p identical to those in which PP5 was shown to be activated by removal of the TPR or C-terminal regions [19]. Under these conditions Ppt1p is also stimulated by lipids toward ^{32}P -MBP, and activating lipids slow TPR domain proteolysis. These results indicate that Ppt1p has a similar domain structure to PP5 and is activated in a similar manner by lipid binding to the TPR domain. Removal of the TPR domain prevents stimulation by lipid, but only modestly increases Ppt1p activity. Carboxyl-terminal truncation modestly increases Ppt1p activity toward both ^{32}P -MBP and ^{32}P -casein, and does not prevent further lipid stimulation of ^{32}P -MBP dephosphorylation. These results distinguish Ppt1p from mammalian PP5 and suggest that the C terminus is less important in controlling Ppt1p activity. Although more work will be required to determine its role in lipid stimulation, removal of the TPR domain had only a small effect on Ppt1p activation. Ppt1p contains two short stretches of amino acids, a 15-residue sequence between the linker helix and the catalytic domain (residues 174–188), and a ten-residue sequence just preceding the extreme C terminus (residues 474–484), that are not found in other PP5 homologs reported thus far ([1–10], and accession numbers CAB60937, CAA17690 and AAD21727). The presence of these additional sequences in Ppt1p may underlie the differences in the responses of Ppt1p and PP5 to N or C-terminal truncation. Additional studies will be required

to determine if our observations reflect structural and regulatory properties unique to Ppt1p.

Ppt1p and PP5 exhibit similar specific activities toward ^{32}P -casein in the absence of lipid. However Ppt1p activity toward this substrate was not stimulated by lipids. These results highlight the fact that PPP enzymes often exhibit substrate specific effects *in vitro* and that the use of a single substrate to study this family of enzymes can be misleading. Like PP5, the control activity of Ppt1p is low toward the commonly used artificial substrates such as ^{32}P -casein and ^{32}P -MBP compared to other PPP enzymes [18]. Thus, it is not clear whether the selective lipid stimulation of Ppt1p toward ^{32}P -MBP reflects a substrate-directed effect, or whether ^{32}P -casein is simply a poor substrate for Ppt1p. Egloff and colleagues identified seven acidic surface residues on the catalytic face of PP1c γ that are replaced by neutral or basic residues in PP2A [25]. They suggest the presence of these acidic residues in PP1c γ may explain why casein is a good substrate for PP2A, but not for PP1. Based on a sequence alignment with PP1c γ , these seven acidic residues are not conserved in Ppt1p. Using the program Modeller [26], a homology model of the catalytic domain of Ppt1p was built based on three PP1 structures – PDB <http://www.pdb.org>[27] codes 1FJM [28], 1IT6 [29] and 1JK7 [30]. This model shows that the catalytic face of Ppt1p is generally less acidic than that of PP1c γ and does not indicate the presence of other acidic residues that could act in the same fashion as those on PP1c γ . Thus the reason for low Ppt1p activity toward this acidic substrate may be more complex than can be explained by electrostatic repulsion.

Others have proposed that fatty acyl coenzyme A esters are potential physiologic regulators of PP5, since these compounds stimulate pNPP hydrolysis by PP5 *in vitro* at physiologically relevant concentrations and are approximately 10-fold more potent than their free fatty acid counterparts [21]. Although we confirmed this observation using PP5 and pNPP (data not shown), in our hands similar levels of AA and arachidoyl coenzyme A were required for half-maximal stimulation of ^{32}P -MBP dephosphorylation in the case of both Ppt1p and PP5. Although more work is required to examine this issue thoroughly, our results suggest that the large differences in potency between free fatty acids and fatty acyl coenzyme A esters seen using pNPP may not extend to all protein substrates. In addition, it will be important to determine if lipids in any form play a role in the regulation of PP5 or Ppt1p *in vivo*.

Ppt1p was present in both the nucleus and cytoplasm of *S. cerevisiae*. No difference in compartmentalization or staining intensity in cells was observed as a function of cell or bud size. This suggests that the level or localization of Ppt1p is not controlled as a function of cell cycle

progression. Both nuclear and cytoplasmic localization have been reported for other PP5 homologs. In some cases, including *P. falciparum* and HeLa cells, nuclear staining appeared more intense than cytoplasmic staining [2,9]. Based on studies with heterologously expressed human PP5-GFP fusion proteins, it has been proposed that nuclear localization requires a stretch of sequence in the C terminus [31]. However, other sequences or signals are also likely to contribute to nuclear localization of PP5 and its homologs, since rat and mouse PP5, which are identical to human PP5 in this region do not appear to be nuclear in all cell types [32,33]. In addition *P. falciparum* PP5 is primarily nuclear [9], although it is not well conserved in the relevant C-terminal region but contains a potential bipartite nuclear localization signal not found in other PP5 sequences reported thus far. The functional significance of differential localization is not known at this time.

Ppt1p levels are highest during periods of rapid growth and drop off when growth slows (Fig. 4B), raising the possibility that this enzyme may have a role in controlling cell growth [34] and its expression may be regulated by a metabolic or nutritional signal [35]. The observation that Ppt1p levels rose and fell in parallel with mRNA levels suggests that Ppt1p expression is primarily controlled at the level of gene transcription. Studies of PP5 and its homologs in cultured cells and various organisms also suggest it may play a role in development [5,6] or during rapid cell growth [2,7].

Conclusions

Ppt1p shares several biochemical properties with PP5 and other homologs, including a similar domain structure and stimulation by lipid *in vitro*. Unlike PP5, Ppt1p is not fully activated by removal of the TPR domain or by removal of 9 C-terminal residues, suggesting that these regions may not play an autoinhibitory role in Ppt1p. Ppt1p is expressed in early log phase growth and decreases dramatically as cells approach the stationary phase, suggesting that Ppt1p could play a role in cell growth or may be controlled by metabolic or nutritional signals. Like PP5, the observation that Ppt1p is localized in both the nucleus and cytoplasm implies that it may function in several sub-cellular compartments.

Although our findings suggest some differences may exist in regulatory properties of Ppt1p and PP5, many biochemical and cellular properties are shared. In addition a recent report has shown that Ppt1p is co-purified with Hsp82p and Hsc82p, the *S. cerevisiae* homologs of hsp90 [36], indicating that Ppt1p and PP5 have at least one common protein partner and suggesting that, like PP5, Ppt1p functions in chaperone complexes. Thus, *S. cerevisiae* Ppt1p is likely to be a valuable model for probing the cellular role of PP5.

Methods

Materials

Restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs, Promega or Gibco BRL. Reagents for bacterial and yeast culture were purchased from Difco. All other materials were from Sigma unless otherwise noted. The yeast centromeric plasmid encoding the Spc42p-EGFP fusion protein was a kind gift from Younghoon Oh and Dr. Harry Charbonneau (Department of Biochemistry, Purdue University).

Cloning and expression of recombinant Ppt1p, Ppt1p (1-504), and PP5 in *E. coli*

PP5 was expressed and purified as described previously [18]. To generate the expression plasmid pET-GST-PPT1, *Saccharomyces cerevisiae* genomic DNA was used as template in a polymerase chain reaction (PCR) with PPT1-specific oligonucleotide primers, (5' primer, 5'-ATGtcaacacccacagcagcagat; 3' primer, 5'-CTAtaaaccaaaccacattagaa) that contained initiation and stop codons, respectively. A PCR product containing the full-length PPT1 coding region was then cloned into the *EcoRV* restriction site of pBII KS - (Stratagene) and sequenced. A fragment containing the PPT1 ORF was excised from the pBII-PPT1 using *EcoRI* and *XhoI* restriction enzymes and was subcloned downstream of the GST coding region into the *EcoRI* and *Sall* sites of the bacterial expression vector pET-21a GST [37]. To construct the pET-GST-PPT1 (1-504) plasmid a 453 bp fragment was amplified from pET-GST-PPT1 by PCR using a 3' primer containing a *XhoI* restriction site and a stop codon designed to truncate the PPT1 gene product at Met504 (5'-gatcctcgagTTAcattggtttatatactggg) and a 5' primer (5'-gtaatgcatggtggtta) encompassing the *NsiI* restriction site of PPT1. The PCR product was cloned into the *NsiI* and *XhoI* restriction sites of pET-GST-PPT1 and sequenced. Each construct was transformed into *E. coli* strain BL21 (DE3) (Novagen), and protein was expressed, purified by glutathione-agarose affinity chromatography, cleaved from GST and stored in 20 mM Tris (pH 7.6 at 4 °C), 50% glycerol, 1 mM EGTA, 0.1% 2-mercaptoethanol, 4 mM MnCl₂ at -20 °C as previously described for PP5 [18]. Recombinant Ppt1p and Ppt1p (1-504) each contain an additional seven residues, Gly-Ser-Gly-Ser-Glu-Phe-Asp, which are remnants of the thrombin-cleavage site. SDS-PAGE and Coomassie stained gels showed that full-length Ppt1p and Ppt1p (1-504) each constituted more than 95% of the purified protein (Fig. 1A).

Phosphatase assays

³²P-labeled substrates were prepared and assays performed as described previously [18]. Briefly, protein phosphatase reactions contained 2 or 4 nM enzyme in a final volume of 30 µl containing buffer A (50 mM Tris, pH 7.6, 1 mM EDTA, 1 mM EGTA, 0.1% 2-mercaptoethanol),

0.4% ethanol and 10 μ M radiolabeled phosphosubstrate in the absence or presence of AA [19]. Reactions were initiated by adding substrate and lipid, and incubated at 30°C for 10 to 15 min. The phosphate released from each substrate was less than 20% of the total present. The amount of ethanol added to lipid-stimulated or control samples had no effect on activity.

Limited proteolysis of Ppt1p

Recombinant protein was incubated with either trypsin (Roche Molecular Biochemicals) or subtilisin (Roche Molecular Biochemicals) in the absence or presence of 250 μ M lipid for 0 to 30 min at 30°C, as previously described [19]. To assess phosphatase activity, an aliquot of the digestion reaction was diluted in buffer A containing either 0.1 mg/ml soybean trypsin inhibitor for trypsin, or 2 mM PMSF for subtilisin and assayed. Matched samples from the proteolysis reaction were quenched with trichloroacetic acid (10%, w/v), protein was sedimented, then boiled in (75 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1% 2-mercaptoethanol and 0.01% bromophenol blue), subjected to SDS-PAGE using 10 or 15% resolving gels, and protein bands visualized by Coomassie staining. For zero time points, samples were withdrawn before the addition of protease but were otherwise identical.

N-terminal sequence analysis of proteolytic fragments

Proteolytic fragments separated by SDS-PAGE were transferred to ProBlott PVDF membranes (Applied Biosystems, Inc.) as described by LeGendre *et al* [38]. Coomassie-stained protein bands were then subjected to sequence analysis using either a Model 491 or Model 470A gas phase sequencer equipped with a Model 120A on-line phenylthiohydantoin analyzer (Applied Biosystems, Inc.). Sequence analysis was performed in the Purdue Macromolecular Structure Facility.

Cloning, subcloning, and gene replacement for the expression of HA-Ppt1p in yeast cells

For expression of HA-tagged Ppt1p under control of the *GAL1* promoter, a *PPT1* DNA fragment was cut from pET-GST-PPT1 by *EcoRI* and *NotI* digestion and subcloned into the *EcoRI/NotI*-digested YCpIF16 (ATCC No. 87091), a centromeric plasmid. The resulting plasmid, YCpIF16-PPT1, was transformed into yeast W303 cells (*MATa*, *can1-100*, *leu2-3,112*, *his3-11,15*, *trp1-1*, *ura3-1*, *ade2-1*). Transformed cells were grown in SC-Trp medium at 30°C to OD_{600 nm} 0.5, washed twice to remove glucose, then grown in SG-Trp medium containing 2% galactose instead of glucose for 12–16 hr at room temperature to induce protein expression.

A strain expressing HA-Ppt1p was made using the two-step gene replacement strategy [39]. First, the upstream sequence of *PPT1* (-955 to -1) was amplified from yeast

genomic DNA by PCR using a 5' primer containing a *KpnI* restriction site (5'-gacGGTACCattagcagcgtgcttaacca-3') and a 3' primer containing a *XhoI* site and HA coding sequence (5'-gacCTCGAGatcgataccgtcgagctagctctgggacgtcgatgggta tcgactcatATTCGAGATATTTGATTATACCAAGGC-3'). In the 3' primer, the sequence from the *XhoI* restriction site to the HA coding sequence was identical to that present in YCpIF16. The amplified DNA fragment was cloned into pRS306 (New England Biolabs, Inc.) using *KpnI* and *XhoI* restriction sites to generate pRS306-955HA. Then, a fragment containing the *PPT1* ORF was excised from YCpIF16-PPT1 by *XhoI* and *NotI* digestion and subcloned into this plasmid to form pRS306-955HAPPT1. The plasmid was linearized by *BamHI* digestion, transformed into the yeast strain, W303, and the transformed cells were plated on a SC-Ura plate. The resulting colonies were then grown in YPAD media (1% yeast extract, 2% peptone, 0.04% adenine, and 2% glucose) and plated on 5-FOA plates to select clones emerging by a second recombination event that eliminated the *URA3* gene together with the additional copy of *PPT1*. To confirm gene replacement, chromosomal DNA from these clones was used for PCR amplification of the region encoding HA-Ppt1p. Amplified product was then analyzed by *XhoI* digest, which can only cut amplified product from the replaced gene.

Immunoblotting and immunocytochemical analysis of HA-Ppt1p expression

Wild type (W303) cell and WHT4-1 cells were grown in YPAD medium. The expression of HA-Ppt1p was detected by immunoblot using the 12CA5 monoclonal anti-HA antibody (Roche Molecular Biochemicals). The total protein was extracted from approximately 10⁷ cells by vortexing with glass beads, then resolved by SDS-PAGE on 12% gel, and transferred to nitrocellulose at 55V in (25 mM Tris, 250 mM glycine, 0.1% SDS, and 20% methanol) for 1 hr at room temperature. The membrane was blocked with 3% non-fat dry milk (Bio-Rad), 0.1% tween-20 in TBS (pH 7.4), then cut at molecular weight marker 45 kDa. The upper half was probed with anti-HA antibody (4 μ g/ml), followed by HRP-conjugated anti-mouse IgG (1:5,000, CalBiochem), and the signal was detected using ECL reagents (Amersham Pharmacia Biotech UK Ltd.). The lower half was probed with anti-Cdc28p antibody (Santa Cruz Biotechnology) to ensure that an equal amount of protein was loaded in each lane. The relative level of HA-Ppt1p expression was determined by comparing the band intensity of HA-Ppt1p to that of Cdc28p using ImageQuant software version 5.1 (Molecular Dynamics).

Cells were prepared for immunocytochemistry as described by Harlow and Lane [40]. Briefly, WHT4-1 cells were grown to early log-phase (OD_{600 nm} 1.0–1.5), fixed

by 4% para-formaldehyde, and the cell wall was removed by treatment with β -glucuronidase and zymolase. The resulting spheroplasts were attached to poly-L-lysine coated coverslips and treated with cold methanol, then acetone. After blocking non-specific binding sites with 3% BSA, 0.1% tween-20 in PBS, the coverslips were incubated with anti-HA (40 μ g/ml) or anti-GFP antibody (40 μ g/ml), rabbit anti-mouse IgG (Pierce, 1:200), biotin-conjugated anti-rabbit IgG (Vector Laboratories, 1:200), and streptavidin conjugated with either Alexa 488 or Alexa 594 (Molecular Probes, 1:200). After staining nuclei with 4'-diamidino-2-phenylindole (DAPI), the coverslips were mounted on slides using ProLong anti-fade reagent (Molecular Probes). The specimens were examined with a BX60 fluorescence microscope (Olympus) and images captured using a RT220 color digital camera (Diagnostic Instruments, Inc.). W303 cells treated in the same manner were used as a negative control.

Northern blot analysis of PPT1 mRNA

Total RNA was prepared from W303 and WHT4-1 yeast cells using hot acid phenol extraction [41]. For electrophoresis, 20 μ g of RNA per sample was resolved on a 1% agarose gel containing 2.2 M formaldehyde at 5V/cm until the bromophenol blue migrated 8 cm. The gel was rinsed with diethylpyrocarbonate-treated H₂O and the RNA was transferred to a Hybond N+ membrane (Amersham Biosciences) by capillary transfer in alkaline solution (0.01 N NaOH, 3 M NaCl) for overnight. The membrane was pre-hybridized with PERFECTHYB PLUS solution (Sigma) at 68°C for 1 hr, and a cDNA probe for PPT1 was added and incubated at 42°C for overnight. The cDNA probe was prepared by restriction digestion of pET-GST-PPT1 with *BsmI* and *NcoI*. The 879 bp DNA fragment generated, corresponding to 544–1422 of PPT1 coding sequence, was gel-purified and labeled with 0.67 μ M [α -³²P] CTP (3000 Ci/mmol) using the DECAprimeII random priming kit (Ambion) according to the user manual. After washing with 1 \times SSC, 0.1% SDS for 20 min at room temperature and 0.2 \times SSC, 0.1% SDS for 20 min three times at 68°C, the membrane was exposed to a storage phosphor screen GP (Kodak) or x-ray film (BMS 1, Kodak). The membrane was stripped in 50% formamide, 0.1 \times SSC, 0.1% SDS for 2 hr at 68°C, and reprobed with a cDNA for ACT1 (yeast actin gene). The DNA fragment corresponding 214–694 of actin coding sequence was amplified by PCR using yeast genomic DNA as template and ACT1-specific oligonucleotide primers (5' primer, 5'-gaacacgggtattgtcaccactgggacgatatgg; 3' primer, 5'-gagcagcgggtttgcattcttctgttcaagtcc). The 481 bp DNA fragment was labeled and used essentially as described for the PPT1 probe. The storage phosphor screen was scanned using Typhoon scanner (Molecular Dynamics), and the relative level of PPT1 message was determined by comparing the band intensity of PPT1 mRNA to that of actin mRNA

using ImageQuant software version 5.1 (Molecular Dynamics).

List of abbreviations

PP, protein phosphatase; MBP, myelin basic protein; TPR, tetratricopeptide repeat; AA, arachidonic acid; HA, hemagglutinin.

Competing interests

None declared.

Authors' contributions

JY cloned, expressed, and performed initial characterization studies of recombinant Ppt1p, performed gene replacement, immunoblot and immunolocalization studies, and prepared the manuscript. JJ and CS characterized PPT1 activity following truncation or limited proteolysis and identified proteolysis sites. JY and JMP carried out Northern blot analyses. SR helped design, supervise and interpret studies and with manuscript preparation. All authors read and approved the final manuscript.

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