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Vacuole Fusion Regulated by Protein Phosphatase 2C in Fission Yeast

Frédérique Gaits and Paul Russell*

Departments of Molecular Biology and Cell Biology, The Scripps Research Institute, La Jolla, California 92037

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The gene *ptc4*$^+$ encodes one of four type 2C protein phosphatases (PP2C) in the fission yeast *Schizosaccharomyces pombe*. Deletion of *ptc4*$^+$ is not lethal; however, Δ*ptc4* cells grow slowly in defined minimal medium and undergo premature growth arrest in response to nitrogen starvation. Interestingly, Δ*ptc4* cells are unable to fuse vacuoles in response to hypotonic stress or nutrient starvation. Conversely, Ptc4 overexpression appears to induce vacuole fusion. These findings reveal a hitherto unrecognized function of type 2C protein phosphatases: regulation of vacuole fusion. Ptc4 localizes in vacuole membranes, which suggests that Ptc4 regulates vacuole fusion by dephosphorylation of one or more proteins in the vacuole membrane. Vacuole function is required for the process of autophagy that is induced by nutrient starvation; thus, the vacuole defect of Δ*ptc4* cells might explain why these cells undergo premature growth arrest in response to nitrogen starvation.

INTRODUCTION

Protein phosphatases that dephosphorylate serine and threonine residues are classified into two super groups (Cohen, 1989). The first group consists of type 1 (PP1), type 2A (PP2A), and type 2B (PP2B) phosphatases, which share ~40% sequence homology in their catalytic domains. These enzymes have multiple subunits, do not require divalent cations, and are sensitive to specific inhibitors such as okadaic acid. The second group consists of the type 2C (PP2C) enzymes. PP2C has no sequence homology to the other group of phosphatases. PP2C is a monomeric enzyme that requires divalent cations (Mg^{2+} or Mn^{2+}) and is insensitive to okadaic acid. Although much is known about the biological functions of PP1, PP2A, and PP2B, the absence of inhibitors and paucity of genetic studies have hindered the analysis of PP2C enzymes.

The understanding of PP2C functions is beginning to improve with the appearance of genetic and cell biology studies that have implicated PP2C in various physiological responses. In mammals and in plants, PP2C appears to be involved in Ca^{2+} signaling (Fukunaga *et al.*, 1993; Leung *et al.*, 1994; Meyer *et al.*, 1994). PP2C also appears to be important for cell maturation and development because its activity is reported to be up-regulated during monocytic differentiation evoked by vitamin D3 in the human leukemic HL-60 cells (Nishikawa *et al.*, 1995). Moreover, a recent study demonstrated that the *FEM-2* gene of *Caenorhabditis elegans* encodes a PP2C enzyme required to promote male development (Chin-Sang and Spence, 1996).

In both the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*, three genes encoding PP2C homologues have been identified (Maeda *et al.*, 1993; Shiozaki and Russell, 1995a,b,c). Mutations in the *TPD1/PTC1* gene of *S. cerevisiae* have pleiotropic effects, including a temperature-sensitive growth defect, failure of cell separation during mitosis, and accumulation of unspliced precursor tRNA species (Robinson *et al.*, 1994). In yeasts and mammals, PP2C has been suggested to negatively regulate stress signals transmitted by stress-activated protein kinases (SAPKs) pathways. These SAPKs include Hog1p in budding yeast, Spc1/StyI in fission yeast, and p38 in mammals (Maeda *et al.*, 1994; Shiozaki *et al.*, 1994, 1995a,b,c; Gaits *et al.*, 1997). It is thought that PP2C might directly dephosphorylate and thereby inactivate SAPKs. Another proposed target of PP2C is the budding yeast kinase Ire1p, located on the endoplasmic reticulum and involved in the regulation of the unfolded protein response via induction of the transcription of endoplasmic reticulum chaperones (Welihinda *et al.*, 1998).

In fission yeast, the three genes that encode PP2C are *ptc1*$^+$, *ptc2*$^+$, and *ptc3*$^+$ (Shiozaki and Russell, 1994, 1995a,b,c). The Δ*ptc1* Δ*ptc2* Δ*ptc3* mutant is viable and retains ~10% of the PP2C activity measured in extracts from wild-type cells, which suggested the existence of at least one other PP2C gene in fission yeast (Shiozaki and Russell, 1995a,b,c). Herein, we describe the initial analysis of *ptc4*$^+$, a fourth PP2C gene in *S. pombe*. Ptc4 is not required for cell viability, but Δ*ptc4* cells exhibit growth defects that are

* Corresponding author. E-mail address: prussell@scripps.edu.

particularly evident during nutrient deprivation. Cells respond to starvation by initiating uptake of cytoplasm into the lysosomal/vacuolar system (Teichert *et al.*, 1989; Bryant and Stevens, 1998). The macromolecules are degraded to produce nutrients necessary to preserve basal metabolism and enhance survival. Our studies suggest that Ptc4 regulates this process, because vacuolar fusion is sensitive to Ptc4 activity and Ptc4 localizes in vacuole membranes.

MATERIALS AND METHODS

Yeast Strains and Media

S. pombe PR109 (*h⁻ leu1–32 ura4-D18*), PR1190 (*h⁻ leu1–32 ura4-D18 his7–366 ade6–216*), FG2341 (*h⁻ leu1–32 ura4-D18 ptc4::ura4⁺*), and FG2340 (*h⁻ leu1–32 ura4-D18 his7–366 ade6–216 ptc4::ura4⁺*) were used for these experiments. Yeast extract medium YES and synthetic minimal medium EMM_2 were used for cell growth. Vacuole visualization was realized by incubation of cells in YSO medium. Growth media and experimental methods for studying fission yeast have been described (Alfa *et al.*, 1993).

Gene Disruption

The one-step gene disruption method was used to construct a *ptc4::ura4* mutant (Rothstein, 1983). A 3.1-kb fragment that contains *ptc4⁺* was PCR-amplified with the 3′ primer CGGCGGCTCGAGGAAGAGAATGCGTGGATG and the 5′ primer CCGCCTCCTGCAGTATGACGGTAGC that contain an *Xho*I and *Pst*I sites, respectively. The PCR product was cloned into the *Eco*RV site of pBluescript-SK (Stratagene, La Jolla, CA). This fragment contains the 1.147-kb coding sequence of *ptc4⁺* as shown in Figure 3. The resulting plasmid was digested with *Cla*I to liberate a 1.2-kb region of *ptc4⁺*. This region was substituted with a 1.8-kb *Hin*dIII fragment of the *S. pombe ura4⁺* gene. The 3.4-kb *Xho*I–*Pst*I fragment that contains *ptc4::ura4⁺* was used for transformation of a diploid strain *h⁻/h⁺ leu1–32/leu1–32 ura4-D18/ura4-D18 ade6-M210/ade6-M216*. Stable Ura⁺ transformants were selected and gene disruption was confirmed by genomic Southern hybridization. After sporulation, phenotypes of the haploid segregants were analyzed.

Purification and Detection of GST-Ptc4 Protein

The coding sequence of *ptc4⁺* was amplified by PCR from the pBSK-*ptc4⁺* vector using the 3′ primer GGAATTCCATATGTCGATCCGTTTTCTTAAACG and the 5′ primer ATAGTTTAGCGGCCGCTTCTTCTGGGATGATAAGC to introduce *Nde*I and *Not*I restriction enzyme sites, respectively. The DNA product was cloned into a pREP1-GST vector to create pREP1-GST-*ptc4⁺*. Wild-type PR109 cells were transformed with the pREP1-GST control vector or the pREP1-GST-*ptc4⁺* vector in which GST-*ptc4⁺* expression was driven by the inducible *nmt1* promoter. Induced or noninduced cells were harvested and lysed in a buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.1% NP40, 10% glycerol, 50 mM NaF, 1 mM Na_3VO_4, 1 μg/ml each of leupeptin, aprotinin, and pepstatin, and 1 mM PMSF. After centrifugation, supernatants were incubated with glutathione (GSH)-Sepharose beads for 2 h at 4°C. The beads were then washed three times in buffer L, and the purified proteins were used to assay phosphatase activity or were resolved by SDS-PAGE and detected by immunoblotting with antisera to GST (generous gift of L. Hengst, TSRI, La Jolla, CA). Immunoreactive bands were revealed with horseradish peroxidase-conjugated secondary antibodies and the ECL Western blotting detection system (Pierce, Rockford, IL).

Analysis of PP2C Activity

GST-Ptc4 purified on GSH-Sepharose beads was used to measure phosphatase activity against phosphorylated casein. Preparation of ^{32}P-labeled casein and procedures of the PP2C phosphatase assay were as described (Cohen, 1989). Okadaic acid (100 nM; Calbiochem, La Jolla, CA) was used to inhibit other serine/threonine-specific phosphatases in the extracts.

Microscopy

For indirect immunofluorescence microscopy, cells were grown to midlog phase at 30°C in EMM_2 medium supplemented with or without 1 mM thiamine. The cells were fixed in −80°C cold methanol and treated for immunofluorescence as described previously (Gaits *et al.*, 1998). Anti-GST antibody was used as a primary antibody to detect GST-Ptc4 and revealed with FITC-conjugated anti-rabbit immunoglobulin G (Zymed, San Francisco, CA) as a secondary antibody. Visualization of vacuoles was performed with live *ade6–216* cells grown overnight in YSO liquid medium at 32°C. Cells were photographed using a Nikon Eclipse E800 microscope equipped with a Photometrics Quantix CCD camera (Nikon, Inc., Melville, NY).

RESULTS

The ptc4⁺ Gene Encodes a PP2C-like Serine–Threonine Phosphatase in S. pombe

In fission yeast, three genes encoding PP2C activity have been cloned: *ptc1⁺*, *ptc2⁺*, and *ptc3⁺*. They account for ~90% of the total PP2C activity detected in cell lysates (Shiozaki *et al.*, 1994, 1995a,b,c). To identify additional PP2C genes, we performed a BLAST search with the sequenced portion of the *S. pombe* genome, using the sequences of Ptc1, Ptc2, and Ptc3. This analysis identified a gene that we named *ptc4⁺* (GenBank accession number for Ptc4 is AF140285). The *ptc4⁺* ORF encodes a 383 amino acid protein with a predicted M_r of 42.2 kDa (Figure 1). Pair-wise sequence comparisons indicate that Ptc2 and Ptc3 are ~51% identical and belong to the same subfamily. Ptc1 and Ptc4 are more divergent (Figure 2).

Recombinant Ptc4 Has PP2C Activity

To examine whether Ptc4 exhibits PP2C-like phosphatase activity, the coding region of *ptc4⁺* was amplified by PCR from the pBSK-*ptc4⁺* vector bearing 3.1 kb of genomic sequence containing the *ptc4⁺* ORF. The amplified fragment was cloned into a pREP1-GST vector that directs expression of Ptc4 with GST fused to its N terminus. In this plasmid, GST-Ptc4 expression was regulated by the thiamine-repressed *nmt1* promoter. The GST-Ptc4 protein was purified from yeast and analyzed by SDS-PAGE after affinity purification on GSH-Sepharose beads. As shown in Figure 3A, a single band with an estimated mass of 70 kDa was detected. The phosphatase activity of GST-Ptc4 was assayed using radioactively labeled phosphorylated casein as substrate. GST-Ptc4, or unfused GST used as a control, were incubated with the substrate with or without 20 mM $MgCl_2$. The ^{32}Pi released in the reaction mixture was measured. Magnesium-dependent casein phosphatase activity was detected with Ptc4 (Figure 3B). GST had no activity. Thus, Ptc4 has all the hallmarks of a type 2C protein phosphatase.

The ptc4⁺ Gene Is Not Essential

To investigate the cellular function of Ptc4, a one-step gene disruption of *ptc4⁺* was performed (Rothstein, 1983). The

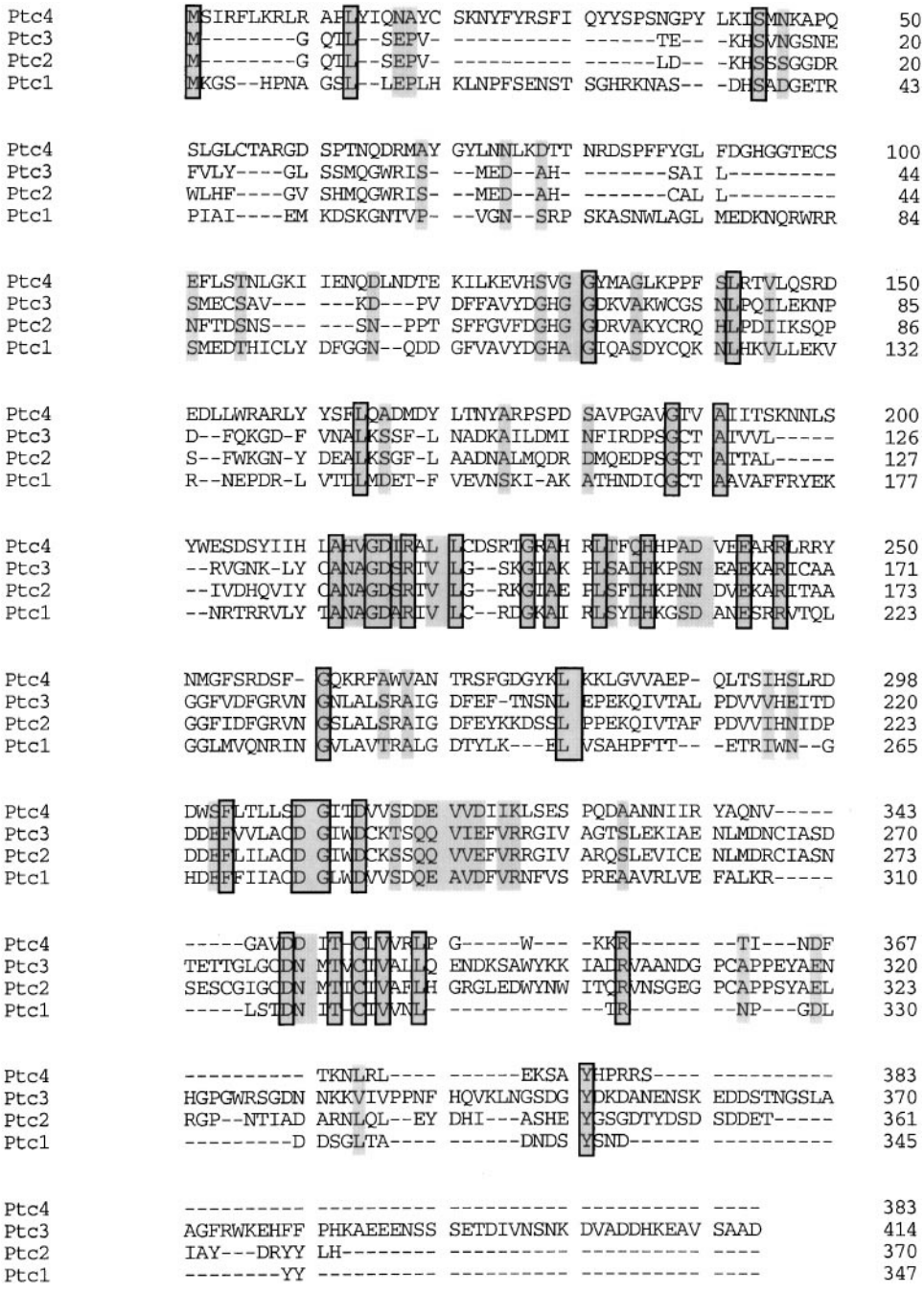

Figure 1. Alignment of the four PP2C proteins in fission yeast.

entire ORF of *ptc4*$^+$ was deleted by substitution with the *S. pombe ura4*$^+$ gene (Figure 4A). A *Xho*I–*Pst*I fragment containing *ptc4*::*ura4*$^+$ was used to replace the *ptc4*$^+$ locus in a diploid strain. Stable Ura$^+$ transformants were selected, and deletion was confirmed by Southern blot analysis (our unpublished data). The heterozygous diploids were sporulated, and the tetrads were dissected. The four spores were viable, and the segregation of the Ura marker was 2$^+$:2$^-$, demonstrating that *ptc4*$^+$ is a nonessential gene. The phenotype of haploid segregants was examined. The Δ*ptc4* cells appeared normal when grown on rich YES medium (Figure 4B); however, when grown in minimal EMM_2 medium, Δ*ptc4* cells were shorter than wild-type cells (Figure 4C). This phenotype was rescued by pREP1-GST-ptc4$^+$, as shown in Figure 4C.

Ptc4 Is Important For Growth in Minimal Medium

Compared with wild-type cells, Δ*ptc4* cells formed small colonies on minimal EMM_2 agar medium (our unpublished

	Ptc1	Ptc2	Ptc3	Ptc4
Ptc1	100%	-	-	-
Ptc2	23%	100%	-	-
Ptc3	21%	51%	100%	-
Ptc4	21%	20%	16%	100%

Figure 2. Pair-wise comparisons of the sequence identity of the four PP2C proteins in fission yeast.

data). We investigated the possibility of a growth defect in liquid EMM_2 medium. As expected, cell growth was dramatically reduced in Δ*ptc4* cells, with normal growth being restored by expression of GST-Ptc4 (Figure 5A). When examined microscopically, Δ*ptc4* cells grown in liquid culture were significantly smaller than wild-type cells.

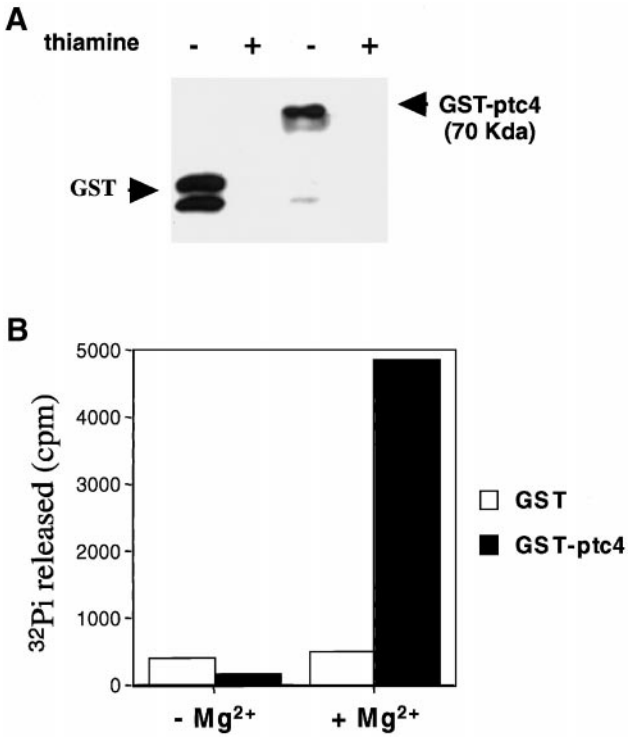

Figure 3. Casein phosphatase activity of Ptc4 protein produced in *S. pombe*. Using GSH-Sepharose beads, GST and GST-Ptc4 fusion proteins were purified from total lysates of wild-type (PR109) cells transformed with pREP1-GST or pREP1-GST-*ptc4*$^+$. Cells were grown for 15 h in thiamine-depleted medium before harvest. (A) Purification of the GST and GST-Ptc4 was confirmed by SDS-PAGE and immunoblotting using anti-GST antibody. (B) GST and GST-Ptc4 were incubated with ^{32}P-labeled casein in the presence or absence of 20 mM $MgCl_2$. Activity is shown as the amount of ^{32}Pi (cpm) released in the reaction mixture.

When *S. pombe* cells experience nutrient limitation, especially nitrogen starvation, they initiate sexual development. This process involves conjugation between cells of opposite mating types (h^- and h^+), meiosis, and finally sporulation. The Δ*ptc4* mutant was partially sterile. The percentage of asci that resulted from mating h^- Δ*ptc4* with h^+ Δ*ptc4* cells was <1%, as compared with ~80% for wild-type cells. Many mating defects can be traced to a failure to arrest in G_1 phase of the cell cycle; therefore, we investigated the behavior of Δ*ptc4* cells under nitrogen starvation. As shown in Figure 5B, wild-type cells cultivated in medium depleted for nitrogen showed a progressive arrest in G_1 phase of the cell cycle. After 6 h, ~20% of wild-type cells arrested with a 1C DNA content. In contrast, Δ*ptc4* cells arrested with a 1C DNA content more quickly than wild-type cells. Approximately 20% of the Δ*ptc4* cells had a 1C DNA content after 3 h, and 60% were arrested in G_1 after 6 h of starvation. This phenotype was completely abrogated by overexpression of GST-Ptc4 (Figure 5B). In fact, GST-Ptc4 overexpression appeared to cause a defect in G_1 arrest. These experiments indicated that the mating defect of the Δ*ptc4* mutant was not caused by a defect in G1 arrest, but might be associated with enhanced sensitivity to nutrient deprivation.

The Δptc4 Cells Are Deficient in Vacuole Fusion

The phenotype of Δ*ptc4* cells was reminiscent of the growth delay and sterility observed in the autophagy-defective mutants of *S. cerevisiae* (Tsukuda and Ohsumi, 1993). Because some of these mutants are defective in components involved in the function of vacuoles, we investigated the role of Ptc4 in the vacuolar system. Differential-interference-contrast (DIC) microscopy was used to compare vacuole morphology of log-phase cells grown in rich medium with stationary phase cells grown in minimal medium (Figure 6A). Wild-type cells grown to stationary phase in minimal medium had several large vacuoles that were easily visible by DIC microscopy. These vacuoles are presumed to result from vacuole fusion as described in *S. cerevisiae* (Teichert *et al.*, 1989; Dunn, 1994). Large vacuoles were not detected in Δ*ptc4* cells grown to stationary phase in minimal medium (Figure 6A).

Hypotonic stress causes transitory fusion of vacuoles in *S. pombe* (Bone *et al.*, 1998). To investigate whether the vacuole fusion defect of Δ*ptc4* cells was nutrient-starvation specific, vacuoles were observed in Δ*ptc4* cells suspended in water. We noticed that vacuoles fluoresced in cells that have the *ade6–216* mutation, which causes the accumulation of a red pigment when they are grown on adenine-poor medium such as YSO. This red pigment apparently accumulates in the vacuoles. In YSO medium, Δ*ptc4* vacuoles appeared consistently smaller and more numerous than in the *ptc4*$^+$ cells (Figure 6B). When cells were collected, washed, and resuspended in water for 10 min, the *ptc4*$^+$ cells had a smaller number of larger vacuoles that resulted from vacuolar fusion (Figure 6B). In contrast, vacuoles remained small and numerous in Δ*ptc4* cells suspended in water (Figure 6B). These findings suggested that Ptc4 regulates vacuole fusion.

Ptc4 Is Localized on the Membrane of Vacuoles and Promotes Vacuolar Fusion

Indirect immunofluorescence was performed to determine the subcellular localization of Ptc4. Wild-type cells were

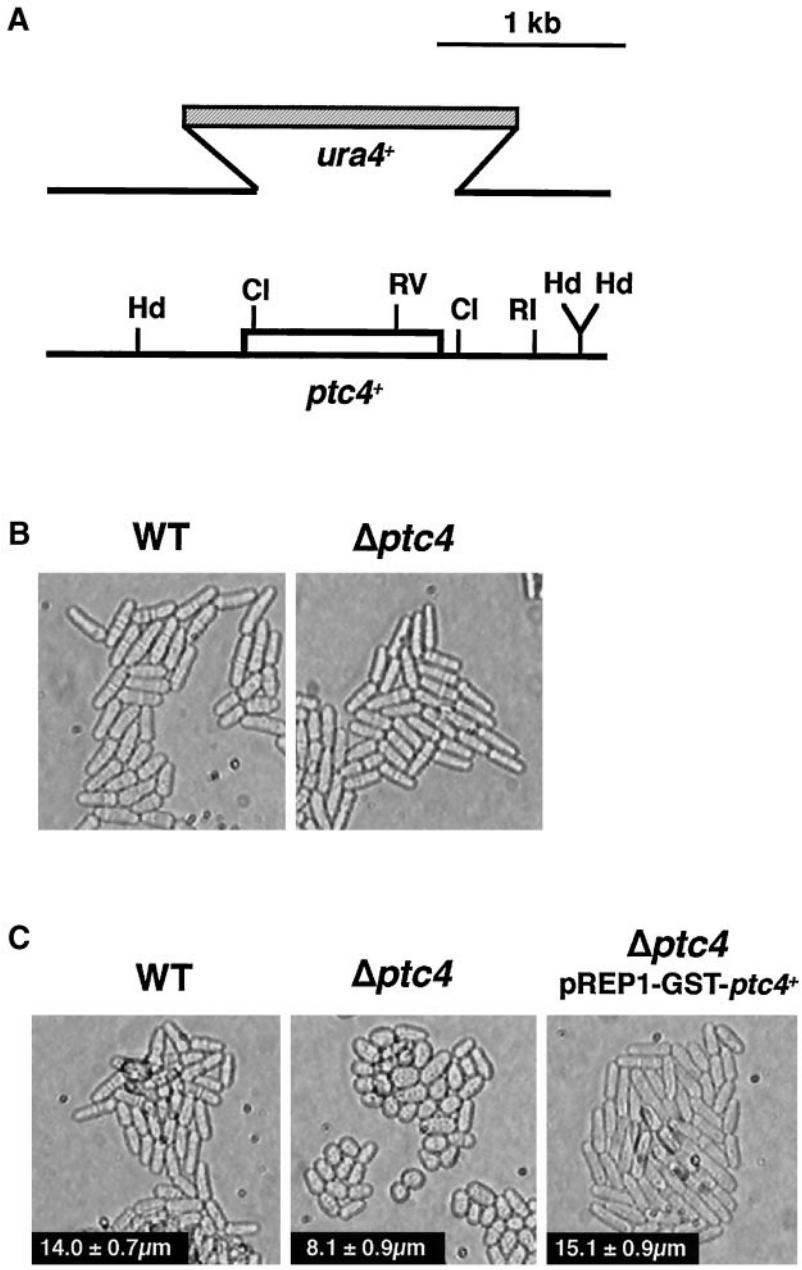

Figure 4. Gene disruption of *ptc4*$^+$. (A) Insertion of the *ura4*$^+$ gene. The pBSK-*ptc4*$^+$ plasmid was cleaved at the *Cla*I sites to substitute the *ptc4*$^+$ ORF with the *ura4*$^+$ gene. The *Xho*I–*Pst*I fragment containing *ptc4*::*ura4*$^+$ was used to transform a diploid strain to replace one chromosomal *ptc4*$^+$ locus by homologous recombination. The transformants were sporulated, and the haploid *ptc4* disruptants were recovered. Hd, *Hin*dIII; Cl, *Cla*I; RV, *Eco*RV; RI, *Eco*RI. (B) Analysis of the *ptc4* disruptant phenotype on YES agar plates. Wild-type (PR109) and Δ*ptc4* cells were plated on YES plates and incubated at 30°C. The phenotypes of the cells were observed after 3 d. (C) Phenotype of Δ*ptc4* on EMM_2 plates. Δ*ptc4* cells were transformed with the pREP1-GST-*ptc4*$^+$. Wild-type (PR109), Δ*ptc4*, and Δ*ptc4*/pREP1-GST-*ptc4*$^+$ were examined after 3 d at 30°C on EMM_2 medium that lacked thiamine.

transformed with pREP1-GST-*ptc4*$^+$ and subsequently treated with anti-GST antibody and FITC-conjugated secondary antibody. When expression was low, under repressed conditions, GST-Ptc4 appeared to localize in vacuole membranes (Figure 6C). This localization was confirmed by examination of cells that expressed green fluorescent protein-tagged Ptc4 from its own promoter (our unpublished data). No similar localization was observed with GST fusions of the other PP2C proteins expressed at a similar level (our unpublished data). Interestingly, when GST-Ptc4 expression was induced by thiamine removal, the number of vacuoles decreased. The decreased number of vacuoles coincided with the appearance of a fewer number of larger vacuoles. After 18 h of induction, >70% of cells showed two or three large vacuolar structures as compared with >30 in wild-type cells (Figure 6). Taken together, these data indicate that Ptc4 is involved in vacuolar fusion in *S. pombe*.

DISCUSSION

In this article, we have cloned and characterized a new gene encoding a member of the PP2C family in *S. pombe*: *ptc4*$^+$. Ptc4 displays the classical Mg^{2+}-dependent phosphatase activity observed with other PP2C proteins in fission yeast (Shiozaki *et al.*, 1994; Shiozaki and Russell, 1995a,b,c). Dis-

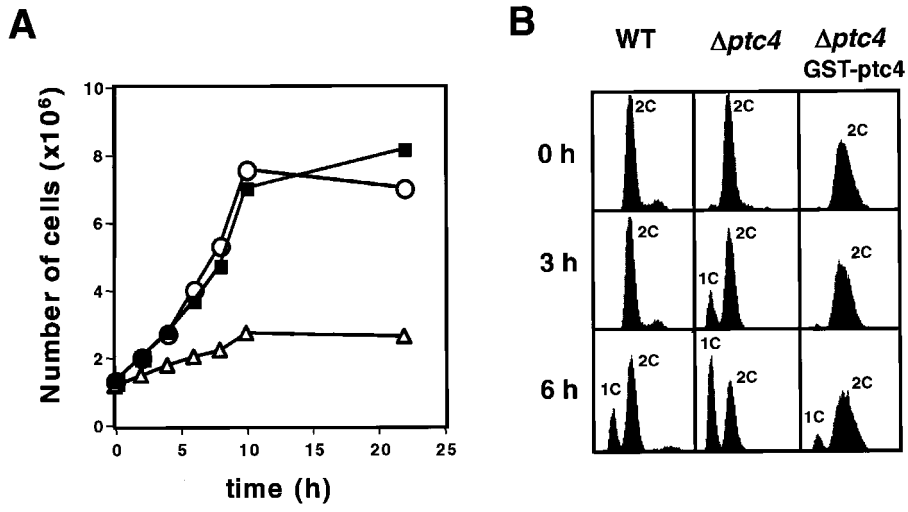

Figure 5. The Δ*ptc4* cells exhibit sensitivity to nutrient limitation. (A) Wild-type (■), Δ*ptc4* (△), or Δ*ptc4* cells expressing GST-Ptc4 (○) were grown in EMM_2 medium at 30°C. Cell density was determined at the indicated time points. (B) FACScan analysis of DNA content in wild-type (PR109), Δ*ptc4*, and Δ*ptc4* cells transformed with pREP1-GST-*ptc4*$^+$ (Δ*ptc4*/GST-ptc4) after nitrogen starvation. Cells grown to midlog phase were switched to minimal medium without nitrogen. After 6 h, 60% of the Δ*ptc4* cells were arrested with a 1C DNA content, whereas only 20% of the wild-type cells had a 1C DNA content. The arrest of Δ*ptc4* cells was rescued by expression of GST-Ptc4.

ruptions of *ptc1*$^+$, *ptc2*$^+$, or *ptc3*$^+$ have minor or undetectable phenotypes, whereas combinatory mutations of PP2C genes generate stress-sensitive phenotypes in both fission and budding yeast (Maeda *et al.*, 1994; Shiozaki and Russell, 1995a,b,c). These observations were attributed to functional redundancy of PP2C enzymes. Interestingly, Δ*ptc4* is the only mutation of fission yeast PP2C genes to cause a substantial phenotype by itself. The Δ*ptc4* cells have a rounded

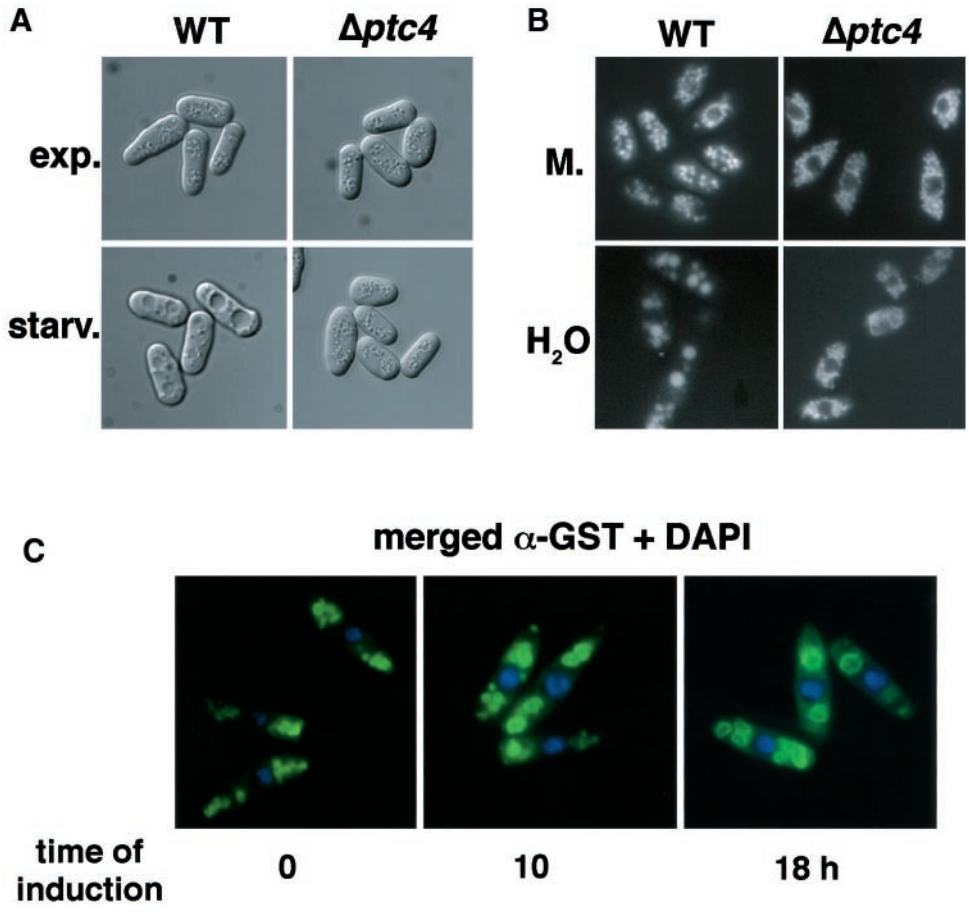

Figure 6. The Δ*ptc4* cells are defective in vacuole fusion. (A) Wild-type (PR109) and Δ*ptc4* cells were grown in rich YES medium (exp.) or in EMM_2 medium to saturation (starv.). Cells were then collected and examined by DIC microscopy. (B) Wild-type (PR1190) and Δ*ptc4 ade6–216* cells were grown in YSO medium, which is poor in adenine. Cells were collected, washed, and resuspended in H_2O. Analysis of vacuoles was performed by fluorescence after 10 min without fixation. (C) GST-Ptc4 is localized on vacuolar membranes. Wild-type cells were transformed with pREP1-GST-*ptc4*$^+$ and grown to midlog phase in EMM_2 medium with thiamine. Cells were then switched to medium without thiamine, and aliquots were harvested and fixed in cold methanol. Cells were incubated with anti-GST antibody to allow detection of GST-Ptc4. Nuclei were visualized with DAPI.

morphology and arrest prematurely when grown in minimal medium. The Δ*ptc4* cells were sensitive to nutrient limitation and were partially sterile. This phenotype is similar to the autophagy-defective mutants in the *S. cerevisiae*. These mutants display a rapid loss of viability under nitrogen starvation associated with sterility (Tsukuda and Ohsumi, 1993). Autophagy is a process conserved throughout evolution from yeasts to mammals. Under conditions of nutrient stress, cells degrade cytosolic macromolecules to produce the elements necessary for their survival. This process is also involved in differentiation when cells remodel intracellular structure. Very little is known about the *S. pombe* autophagy, and the nature of the proteins involved is still unclear. Whether PP2C phosphatases play a role in autophagy regulation remains to be determined (Dunn, 1994; Bryant and Stevens, 1998).

Experiments were performed to investigate the vacuolar system in Δ*ptc4* cells. When grown in rich medium, Δ*ptc4* cells displayed a large number of small vacuoles that were comparable to vacuoles in wild-type cells grown in similar conditions. However, when grown to stationary phase in minimal medium or resuspended in water, Δ*ptc4* cells failed to display the vacuolar fusion that is observed in wild-type cells. In *S. cerevisiae*, the vacuolar morphology led to division of the mutants into six classes (A to F). On the basis of microscopic observation, Δ*ptc4* is closely related to the class B mutants, which display a large number of highly fragmented vacuoles (Banta *et al.*, 1988). Subcellular localization of the fusion protein GST-Ptc4 demonstrated that Ptc4 was associated with the membranes of vacuoles. When highly overexpressed, GST-Ptc4 induced vacuole fusion. Taken together, these data suggest that Ptc4 is involved in the regulation of vacuolar fusion. Presumably, one or more proteins that regulate vacuole fusion are regulated by phosphorylation. These could be proteins that promote vacuolar fusion and are inhibited by phosphorylation, or proteins that negatively regulate fusion and are activated by phosphorylation. Vacuole fusion has been shown to require phosphatase activity. Indeed, microcystin-LR, a potent inhibitor of type 1 and 2A serine/threonine phosphatases, inhibits the fourth step of the vacuole inheritance reaction in vitro, which corresponds to the fusion step (Conradt *et al.*, 1994; Mayer and Wickner, 1997); however, the activity involved in these experiments does not correspond to Ptc4 activity because type 2C phosphatases are insensitive to microscystin-LR. Several kinases appear to be involved in vacuolar signaling and sorting, such as the SAPK Spc1/StyI in fission yeast and the PI3-kinase homologue Vsp34 in budding yeast (Schu *et al.*, 1993; Takegawa *et al.*, 1995; Bone *et al.*, 1998). Spc1 is required for vacuole fusion (Bone *et al.*, 1998). Like all SAPKs, Spc1 is activated by a SAPK kinase, in this case Wis1 (Millar *et al.*, 1995; Shiozaki and Russell, 1995a,b,c); however, it appears unlikely that Ptc4 dephosphorylates Spc1, because loss of Ptc4 would be expected to enhance Spc1 activity and not impair vacuole fusion. Likewise, these data cannot be easily explained by the proposition that PP2C dephosphorylates substrates of Spc1. These assumptions are consistent with the apparent absence of Spc1 in vacuolar membranes (Gaits *et al.*, 1998), although it is possible that Spc1 phosphorylates proteins that subsequently associate with vacuole membranes. Vps34 is mostly a phosphatidylinositol-specific PI 3-kinase; however, it is able to autophosphorylate. Vps34 is found at the vacuolar membrane associated with a protein serine/threonine kinase, Vps15, which is required for Vps34 lipid kinase activity (Stack *et al.*, 1995). Ptc4 might dephosphorylate the substrate of Vps15.

Further investigation of the role of Ptc4 in vacuolar organization and growth adaptation during nutrient starvation, specifically by identifying its targets and regulators, may lead to a better understanding of the autophagy process in *S. pombe*. In addition, it may provide a more general understanding of the role of phosphatases in membrane and organelle plasticity.

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