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# Protein Phosphatase 2C, Encoded by *ptc1*$^+$, Is Important in the Heat Shock Response of *Schizosaccharomyces pombe*

KAZUHIRO SHIOZAKI, HALEH AKHAVAN-NIAKI, CLARE H. McGOWAN, AND PAUL RUSSELL*

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

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**Protein phosphatase 2C (PP2C), an $Mg^{2+}$-dependent enzyme that dephosphorylates serine and threonine residues, defines one of the three major families of structurally unrelated eukaryotic protein phosphatases. Members of the two other families of protein phosphatases are known to have important cellular roles, but very little is known about the biological functions of PP2C. In this report we describe a genetic investigation of a PP2C enzyme in the fission yeast *Schizosaccharomyces pombe*. We discovered *ptc1*$^+$ (phosphatase two C) as a multicopy suppressor gene of *swo1-26*, a temperature-sensitive mutation of a gene encoding the heat shock protein hsp90. The *ptc1*$^+$ gene product is a 40-kDa protein with ~24% identity to a rat PP2C protein. Purified Ptc1 has $Mg^{2+}$-dependent casein phosphatase activity, confirming that it is a PP2C enzyme. A *ptc1* deletion mutant is viable and has approximately normal levels of PP2C activity, observations consistent with the fact that *ptc1*$^+$ is a member of a multigene family. Although a *ptc1* deletion mutant is viable, it has a greatly reduced ability to survive brief exposure to elevated temperature. Moreover, *ptc1*$^+$ mRNA levels increase 5- to 10-fold during heat shock. These data, demonstrating that Ptc1 activity is important for survival of heat shock, provide one of the first genetic clues as to the biological functions of PP2C.**

Eukaryotic protein phosphatases have traditionally been classified into five broad classes on the basis of substrate amino acid specificity and cofactor requirements (2). However, gene cloning and sequencing studies have revealed that there are in fact three major evolutionary families of eukaryotic protein phosphatases. One family includes members that dephosphorylate exclusively tyrosine together with the more recently recognized dual-specificity phosphatases such as Cdc25 that dephosphorylate tyrosine and threonine in vivo (reviewed in reference 25). This is a highly divergent family, but all of its members share a conserved active site that includes a cysteine residue that is the phosphate acceptor in the dephosphorylation reaction. The second major family consists of type 1 (PP1), type 2A (PP2A), and type 2B (PP2B) serine/threonine phosphatases (2). These enzymes share ~40% identity of amino acid sequence in their catalytic domains, show sensitivity to certain phosphatase inhibitors such as okadaic acid, and do not require $Mg^{2+}$ for enzymatic activity (2). A number of new phosphatase genes whose protein products have a structural similarity to this family have been identified in a variety of species (4), and the family continues to expand.

The third major protein phosphatase family is made up of type 2C enzymes (PP2C). Like the second family, these enzymes predominantly dephosphorylate serine and threonine residues, but their activity is $Mg^{2+}$ dependent and resistant to okadaic acid (2). These enzymes are ubiquitous in eukaryotes; PP2C activity has been detected in a variety of species, and PP2C genes have been cloned in rats (35, 36), rabbits, humans (19), and most recently in the budding yeast *Saccharomyces cerevisiae* (18). PP2C enzymes share ~25% identity in their catalytic domains, but they have no detectable sequence similarity to the phosphatases from the other families.

Genetic studies have been instrumental in unraveling the biological functions of various members of the tyrosine phosphatases and PP1/PP2A/PP2B serine/threonine phosphatases. In addition to functions in basic processes of cellular metabolism, these phosphatases have important roles in cell cycle control, as well as regulation of cell proliferation and differentiation. In contrast, very little is known about physiological functions of PP2C, mainly because of a lack of genetic studies or a specific inhibitor of PP2C. Here we report the cloning and characterization of a PP2C gene in *Schizosaccharomyces pombe*. This gene, *ptc1*$^+$, was isolated as a multicopy suppressor of *swo1-26*, a temperature-sensitive mutation of a gene encoding an 82-kDa protein that is closely related to the stress protein hsp90. *ptc1*$^+$ mRNA is heat inducible, and *ptc1* deletion mutant cells are highly sensitive to a brief heat shock. Protein phosphorylation is believed to regulate many of the dramatic cellular changes that occur during stress, but little is known about the protein kinases and phosphatases that mediate this regulation. Our data demonstrate that the PP2C enzyme encoded by *ptc1*$^+$ has a significant role in the heat shock response.

## MATERIALS AND METHODS

***S. pombe* strains and media.** Haploid strains of *S. pombe* used were derivatives of *972h*$^-$ and *975h*$^+$ (26). PR109 (*h*$^-$ *leu1-32 ura4-D18*) was used as a wild-type strain. Isolation of *swo1-26* will be described elsewhere (1). YES and synthetic EMM2 media (28) were used in growing *S. pombe* cells.

**Cloning of *ptc1*$^+$.** PR166 (*h*$^-$ *swo1-26 ura4-D18 leu1-32*) cells were transformed with an *S. pombe* genomic library by using the pFL20 vector (9), which contains *S. cerevisiae URA3* as a selectable marker. Transformation was performed by the lithium method (12). The plasmids recovered from Ts$^+$ Ura$^+$ transformants were further analyzed by restriction enzyme mapping. Three of those plasmids turned out to be overlapping and contained *ptc1*$^+$. One of them, pFL20-24, was used for subcloning. An *S. pombe* cDNA library made in pUC8 vector was screened with the *Bam*HI-*Xho*I 3.0-kb fragment of pFL20-24 as a probe, and a 1.9-kb *ptc1*$^+$ cDNA was recovered.

The *ptc1*$^+$ open reading frame (ORF) sequence was amplified by PCR with *ptc1*$^+$ cDNA (described above) as a template.

* Corresponding author. Phone: (619) 554-8273. Fax: (619) 554-6165.

The 5′ oligonucleotide 5′CCGAATTCATATGAAGGGAAG CCATCCA3′ containing *Eco*RI and *Nde*I sites hybridized to the sequence surrounding the ATG initiation codon, whereas the 3′ oligonucleotide 5′TTAAGCTTGCGGCCGCAATAGT CATTACTGTATGAATC3′ containing *Not*I and *Hin*dIII sites hybridized to the sequence surrounding the TAG termination codon. A PCR-amplified 1.1-kb fragment was ligated downstream of the thiamine-repressible *nmt1* promoter (20) in a modified pREP1 plasmid, pREP1-Ha6H (37), after digestion with *Nde*I and *Not*I to form pREP1-ptc1Ha6H.

**Gene disruption.** To construct a *ptc1* disruptant, the one-step gene disruption method (31) was employed. Disruption of *ptc1* was performed by two different procedures, creating an insertion in one case and a deletion in the other. For insertion, a 1.6-kb *Bgl*II fragment of the *S. pombe ura4*$^+$ gene was inserted at the *Bam*HI site of a 3.7-kb *Eco*RI-*Hin*dIII *ptc1*$^+$ fragment cloned in pTZ19 (Pharmacia). The resulting plasmid was digested with *Eco*RI and *Hin*dIII to liberate the *ptc1*::*ura4*$^+$ fragment and used for transformation of diploid *h*$^+$/*h*$^+$ *leu1-32*/*leu1-32 ura4-D18*/*ura4-D18* cells. Stable transformants were obtained, and the phenotypes of their haploid segregants were examined. Genomic DNA was isolated from the heterozygous diploids and their segregants and subjected to PCR and Southern hybridization analysis to confirm that *ptc1*::*ura4*$^+$ had replaced *ptc1*$^+$ in the chromosome by homologous recombination. To create a deletion mutation, the *Sph*I 2.4-kb genomic fragment of *ptc1*$^+$ was used. The 1.5-kb *Bgl*II-*Sal*I region was substituted with a 2.8-kb *Bgl*II-*Sal*I *LEU2* fragment. The 3.7-kb *Sph*I fragment containing *ptc1*::*LEU2* was used for transformation of *S. pombe* OM357 (*h*$^+$ *leu1-32 ura4-D18 ptc1*::*ura4*$^+$). Stable Leu$^+$ Ura$^-$ transformants were then selected, and genomic Southern hybridization analysis confirmed the deletion of whole *ptc1* ORF.

**Expression of Ptc1 protein in *Escherichia coli*.** The *ptc1*$^+$ ORF 1.1-kb fragment amplified by PCR as described above was digested by *Eco*RI and *Hin*dIII and ligated into pGEX-KG (8) to form pGEX-KG-ptc1. *E. coli* DH5α cells were transformed with this plasmid, and GST-Ptc1 fusion protein was isolated from the cell lysate by using glutathione (GSH)-Sepharose beads (Pharmacia) as previously described (24).

**Phosphatase assay.** Preparation of substrate, $^{32}$P-labeled casein, and procedures for assay were described by McGowan and Cohen (23). For assaying PP2C activity in *S. pombe* extracts, 100 nM okadaic acid (Calbiochem) was added to the reaction mixture to inhibit PP2A activity.

**Fractionation of *S. pombe* crude extracts by gel filtration or MonoQ chromatography.** *S. pombe* cells were grown in YES medium to mid-log phase and harvested by centrifugation. All procedures described below were done at 4°C. For gel filtration fractionation, cells were washed with lysis buffer (50 mM triethanolamine [pH 7.5], 1 mM EDTA, 200 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 0.1% 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) and disrupted with glass beads in the same buffer. The homogenate was centrifuged at 55,000 rpm for 20 min in a Beckman TLA100.2 rotor. All centrifugations described below were done with the same rotor. The supernatant was filtrated by a 0.2-μm-pore-size filter (Acrodisc, Gelman Sciences) and then applied to a Superose 12 HR10/30 column (Pharmacia). Proteins were eluted by the lysis buffer at the flow rate of 0.2 ml/min, and fractions of 0.5 ml were collected for phosphatase assay. The column was calibrated by an additional run with marker proteins (apoferritin, alcohol dehydrogenase, and ovalbumin [Sigma]) under exactly the same conditions. For fractionation by MonoQ chromatography, cells were washed with TEG buffer (50 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10% glycerol, 1 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) containing 400 mM NaCl and disrupted in the same buffer. After centrifugation at 40,000 rpm for 20 min, the supernatant was recovered and diluted with TEG buffer such that the NaCl concentration was 100 mM. The extract was centrifuged again at 55,000 rpm for 20 min, and the supernatant was chromatographed on a MonoQ HR5/5 column (Pharmacia) in a 20-ml linear gradient between 100 and 500 mM NaCl and then in a 7-ml gradient to 1 M NaCl in Q buffer (20 mM triethanolamine [pH 7.5], 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol). Fractions (1 ml) were collected, and phosphatase activity was measured.

**Heat shock experiments.** Cells were grown in YES medium to mid-log phase at 25°C. Cultures to be pretreated were transferred to a 37°C water bath for 30 min. A sample of 1 ml of the cultures with or without pretreatment was inoculated into 9 ml of YES medium prewarmed at 48°C at time zero. Every 5 min, an aliquot was taken and diluted with ice-cold YES medium and then plated onto YES solid medium. The plates were incubated at 25°C, and the number of colonies was counted after 6 days. Experiments were repeated at least twice for each strain.

**Other general methods.** Southern and Northern (RNA) hybridization analyses were performed as described by Sambrook et al. (32). The method of Laemmli (15) was used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and proteins were detected by Coomassie brilliant blue R250 (Sigma) staining.

**Nucleotide sequence accession number.** The nucleotide sequence presented here has been assigned EMBL accession number L26970.

## RESULTS

**The *ptc1*$^+$ gene encodes a PP2C-like protein in *S. pombe*.** *ptc1*$^+$ was isolated as a multicopy suppressor of the *swo1-26* mutation. *swo1-26* (suppressor of Wee1 overproduction) was originally identified as a suppressor mutation which can rescue the lethal phenotype of overproduction of a mitotic inhibitor, Wee1 (1). The *swo1*$^+$ gene encodes an 82-kDa protein which shares about 60% identity to hsp90 in higher eukaryotes. Mutant *swo1-26* cells are approximately normal at 25°C but are inviable at 35.5°C. These cells were transformed with an *S. pombe* genomic library, and plasmids which could rescue the temperature-sensitive phenotype were recovered (Materials and Methods). Five clones were found to complement *swo1-26* reproducibly, and restriction enzyme mapping of those clones revealed that three of them were overlapping. One of them, pFL20-24, contained a 9.5-kb genomic fragment as an insert. Complementation tests with subclones pFL20-24a and pFL20-24b (Fig. 1A) indicated that the region essential for rescue of the *swo1-26* temperature-sensitive phenotype included the *Bam*HI site, but pFL20-24c containing *Bgl*II-*Xho*I 3.8-kb fragment failed to rescue. To test whether the ORF of the gene responsible for complementation encompassed the *Bgl*II site, we digested pFL20-24 with *Bgl*II and religated after blunting the DNA with Klenow polymerase fragment. This treatment adds four nucleotides and thus is expected to cause a shift of the reading frame if the *Bgl*II site is in the ORF. The resulting plasmid, pFL20-24d, did complement *swo1-26*, which suggests that the *Bgl*II site is outside the ORF but probably in a region important for expression of the ORF. Therefore, we screened an *S. pombe* cDNA library by using the *Bam*HI-*Xho*I 3.0-kb fragment of pFL20-24 as a probe. Two positive clones contained an identical 1.9-kb insert, and nucleotide sequencing identified a 1,041-bp ORF encoding a 347-amino-acid 40-kDa

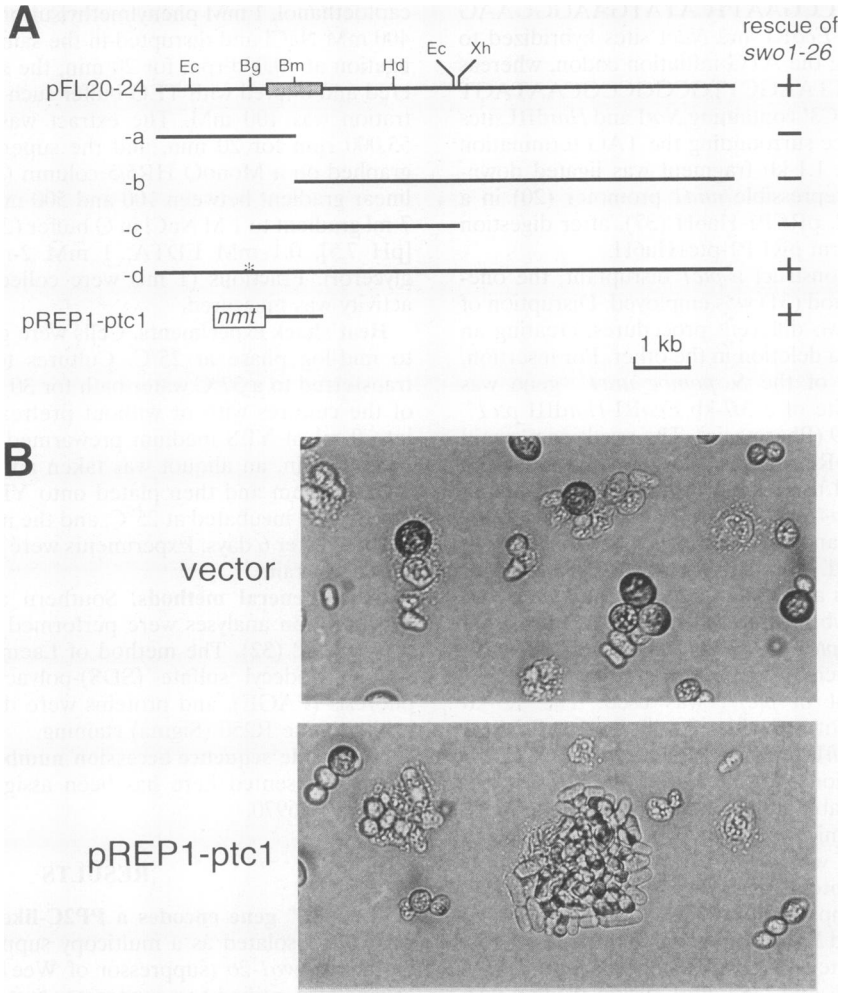


FIG. 1. Cloning of the *ptc1*$^+$ gene. (A) Subcloning of *ptc1*$^+$ genomic clone. pFL20-24 containing *ptc1*$^+$ (ORF is indicated by a shaded box) rescues the growth defect of *swo1-26* mutant cells at 35.5°C. pFL20-24 derivatives having deletions (-a, -b, and -c) or the disrupted *Bgl*II site (shown by an asterisk in derivative -d) were examined for their abilities to rescue *swo1-26*. The plasmid pREP1-ptc1 carrying the *ptc1*$^+$ ORF under the control of *nmt1* promoter (20) complemented the *swo1-26* mutation in thiamine-depleted media. Bg, *Bgl*II; Bm, *Bam*HI; Ec, *Eco*RI; Hd, *Hin*dIII; Xh, *Xho*I. (B) Rescue of *swo1-26* by overproduction of Ptc1. PR166 (*h*$^-$ *leu1-32 ura4-D18 swo1-26*) cells were transformed with the vector pREP1 (vector) or pREP1-ptc1. The transformants were patched from selective media containing thiamine onto thiamine-depleted plates. After 18 h of incubation at 25°C, the plates were transferred to 35.5°C, and cells were examined for phenotype by a phase-contrast microscope.

protein (Fig. 2). The DNA fragment corresponding to the putative ORF was amplified by PCR and ligated downstream of the thiamine-repressible *nmt1* promoter (20). The *swo1-26* cells carrying this construct on a multicopy plasmid could grow and form colonies at 35.5°C on media lacking thiamine (Fig. 1B, lower panel). On the other hand, the same strain transformed with control pREP1 vector lacking the ORF stopped dividing after a shift to a restrictive temperature (Fig. 1B, upper panel). The cells gradually became swollen, and many eventually lysed. These results indicated that this ORF carries the complementation activity.

A data base search identified similarity between the predicted amino acid sequence of the ORF and mammalian PP2Cs (Fig. 3). Therefore, we named this newly isolated gene *ptc1*$^+$ (for phosphatase two C). Rat PP2Cα and PP2Cβ are 24 and 21% identical to *ptc1*$^+$, respectively (35, 36). Recently, a PP2C homolog from the budding yeast *S. cerevisiae*, *PTC1*, was also reported (18). *S. pombe ptc1*$^+$ shares 35% identity with *PTC1* (Fig. 3).

***ptc1*$^+$ protein has a phosphatase activity similar to that of mammalian PP2C.** To examine whether Ptc1 protein has a PP2C-like phosphatase activity, *ptc1*$^+$ was expressed in *E. coli* as a fusion protein containing GSH *S*-transferase (GST) attached to the N terminus. The GST-Ptc1 protein was purified by being absorbed to GSH-Sepharose beads (34). A single polypeptide band with an estimated mass of 60 kDa was seen by SDS-PAGE (data not shown). Phosphatase activity of this fusion protein was assayed by using phosphorylated casein as a substrate (23). GST-Ptc1 bound to GSH-Sepharose was incubated in the reaction mixture with or without 20 mM $MgCl_2$, and the amount of released $^{32}P_i$ was measured (Fig. 4). GST-Ptc1 had high casein phosphatase activity that was dependent on magnesium, whereas no activity was detected in the control reaction mixture with unfused GST. A similar experiment was carried out with Ptc1 protein expressed in *S. pombe*. Ptc1 protein tagged with six consecutive histidine residues at the C terminus was overproduced under the control of *nmt1* promoter and purified on Ni-nitrilotriacetic acid-

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                                                                                                    CCAAGGAAAATCGGTGTTTCTCGTATA  -101
TACTATACAATCAGCAATTTTATACCTGATATTATAATTGAAGGTATTTAGTTAATTTGAAAAGCAATTTTTTTTGTCGGTACTGAAGTGTTTGAACGAAT    -1

ATG AAG GGA AGC CAT CCA AAT GCA GGT TCC CTG TTG GAA CCA CTT CAC AAG TTG AAC CCA TTT TCT GAA AAT TCT    75
M  K  G  S  H  P  N  A  G  S  L  L  E  P  L  H  K  L  N  P  F  S  E  N  S     25

ACA AGT GGA CAT CGA AAA AAC GCT AGT GAT CAT TCG GCG GAT GGA GAA ACT CGA CCG ATT GCA ATT GAG ATG AAG   150
T  S  G  H  R  K  N  A  S  D  H  S  A  D  G  E  T  R  P  I  A  I  E  M  K     50

GAT AGT AAA GGC AAC ACA GTC CCA GTT GGT AAC TCG CGC CCT AGT AAA GCC TCT AAC TGG CTT GCT GGA CTG ATG   225
N  S  R  P  S  K  A  S  N  W  L  A  G  L  M  E  D  K  N  Q  D  S  K  G  N     75

GAG GAT AAA AAC CAA AGA TGG CGT AGG TCT ATG GAG GAT ACT CAT ATT TGC TTG TAT GAT TTT GGG GGT AAT CAG   300
T  V  P  V  G  R  W  R  R  S  M  E  D  T  H  I  C  L  Y  D  F  G  G  N  Q    100

GAC GAC GGC TTC GTT GCC GTT TAT GAC GGA CAT GCT GGC ATT CAA GCA TCT GAC TAC TGT CAG AAG AAT CTT CAC   375
D  D  G  F  V  A  V  Y  D  G  H  A  G  I  Q  A  S  D  Y  C  Q  K  N  L  H    125

AAG GTA CTT TTG GAA AAG GTT AGG AAT GAG CCG GAC CGG CTG GTC ACA GAC CTA ATG GAC GAA ACA TTT GTC GAG   450
K  V  L  L  E  K  V  R  N  E  P  D  R  L  V  T  D  L  M  D  E  T  F  V  E    150

GTC AAC AGC AAA ATT GCA AAG GCT ACT CAC AAT GAC ATT TGC GGT TGT ACC GCT GCA GTT GCT TTC TTT AGA TAT   525
V  N  S  K  I  A  K  A  T  H  N  D  I  C  G  C  T  A  A  V  A  F  F  R  Y    175

GAG AAA AAC CGC ACC CGC CGT GTT CTC TAC ACA GCT AAT GCA GGT GAT GCA CGT ATC GTA TTA TGC CGT GAT GGT   600
R  R  V  L  Y  T  A  N  A  G  D  A  R  I  V  L  C  R  D  G  E  K  N  R  T    200

AAG GCT ATT CGA CTT TCT TAC GAT CAC AAG GGA TCC GAT GCC AAT GAG TCT CGT CGT GTG ACC CAG TTG GGT GGT   675
K  A  I  R  L  S  Y  D  H  K  G  S  D  A  N  E  S  R  R  V  T  Q  L  G  G    225

CTC ATG GTA CAA AAC CGA ATT AAT GGC GTT CTT GCT GTC ACA AGG GCC TTA GGT GAT ACC TAT CTT AAG GAA TTG   750
L  M  V  Q  N  R  I  N  G  V  L  A  V  T  R  A  L  G  D  T  Y  L  K  E  L    250

GTC AGT GCG CAT CCC TTC ACC ACT GAA ACC CGA ATC TGG AAC GGT CAT GAT GAG TTC TTC ATT ATC GCT TGT GAT   825
V  S  A  H  P  F  T  T  E  T  R  I  W  N  G  H  D  E  F  F  I  I  A  C  D    275

GGC TTG TGG GAT GTA GTA TCG GAT CAG GAA GCT GTC GAC TTT GTT CGC AAT TTC GTA AGC CCT CGC GAA GCC GCT   900
G  L  W  D  V  V  S  D  Q  E  A  V  D  F  V  R  N  F  V  S  P  R  E  A  A    300

GTT CGC CTG GTA GAA TTT GCA TTA AAA CGT CTT AGT ACA GAC AAT ATC ACT TGT ATT GTA GTT AAC TTG ACT CGC   975
V  R  L  V  E  F  A  L  K  R  L  S  T  D  N  I  T  C  I  V  V  N  L  T  R    325

AAT CCC GGT GAT CTC GAT GAT TCA GGA CTT ACC GCT GAT AAT GAT TCA TAC AGT AAT GAC TAT TAT TAG GTATTTG  1051
N  P  G  D  L  D  D  S  G  L  T  A  D  N  D  S  Y  S  N  D  Y  Y  *             347

ATGGATTCAATGATGTGCTTTCTGTTGTTTCGTAACGGTTTTTCGATGTCTTTACCTCATCCACAGTACTTGTTTGGAAGCCGGAATGACTTAATCCTA  1150
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TAAATCTGTCCAAATCTAGTTTCTTTTTTTCTTTTTTTCATGATTTGCTACCTTTTCCTCCAATCTGCGTGCCACTGTTCGAATCAATCGTATATTCATCA  1447
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GGGTATTGGCATTCGCAAATATTATGAATGTACTTGCGTGCATGCATTGGTGTTTATAACAAAGGCTATACGTTTCACCAAAAAAAATTTATTTTACCC  1645
TTTTATCGTCTTTAATTTTCGATTTAAAAGTCGAATTATAATTATTATTCTGAAGTTACGGGTTGTCTTTATTTTGTCCCGTTCCTGCTAAAAAAAAAA  1744
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                                        1788
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FIG. 2. Nucleotide sequence of *ptc1*$^+$ cDNA and predicted amino acid sequence. The ORF encodes a 347-amino-acid protein with a calculated molecular mass of 40 kDa.

agarose beads (10). Magnesium-dependent casein phosphatase activity was detected also with this protein expressed in *S. pombe* (data not shown). These results indicated that Ptc1 protein has a serine/threonine protein phosphatase activity similar to that of mammalian PP2C.

**A *ptc1* deletion mutant is viable.** The cellular function of *ptc1*$^+$ was investigated by creating a *ptc1* null mutant. The *ptc1*$^+$ gene was cleaved at the *Bam*HI site, and an *S. pombe ura4*$^+$ gene fragment was inserted (Fig. 5A). The plasmid was linearized and used for one-step gene replacement (31) of chromosomal *ptc1*$^+$ gene in a diploid strain. Stable Ura$^+$ transformants were selected, and the gene disruption was confirmed by PCR and Southern hybridization (data not shown). The heterozygous diploid was sporulated, and the phenotypes of haploid segregants were examined. Ura$^+$ haploid cells were viable and grew normally at 20 to 36°C, which suggested that *ptc1*$^+$ is a nonessential gene.

To be certain that *ptc1*$^+$ is nonessential, we deleted the entire ORF of *ptc1*$^+$ and found that the mutant cells again showed no apparent growth defect. The 3.2-kb *Bgl*II-*Sal*I region of *ptc1*$^+$ gene fragment was substituted with *S. cerevisiae LEU2* (Fig. 5B). This plasmid was used for transformation of a haploid Ura$^+$ segregant described above, in which genomic *ptc1*$^+$ gene has a *ura4*$^+$ insertion. Genomic Southern hybridization analysis of Leu$^+$ Ura$^-$ transformants confirmed the complete deletion of *ptc1*$^+$ (Δ*ptc1*).

Tetrad analysis of a cross between *swo1-26* and the deletion mutant Δ*ptc1* showed no linkage of these two loci. The double

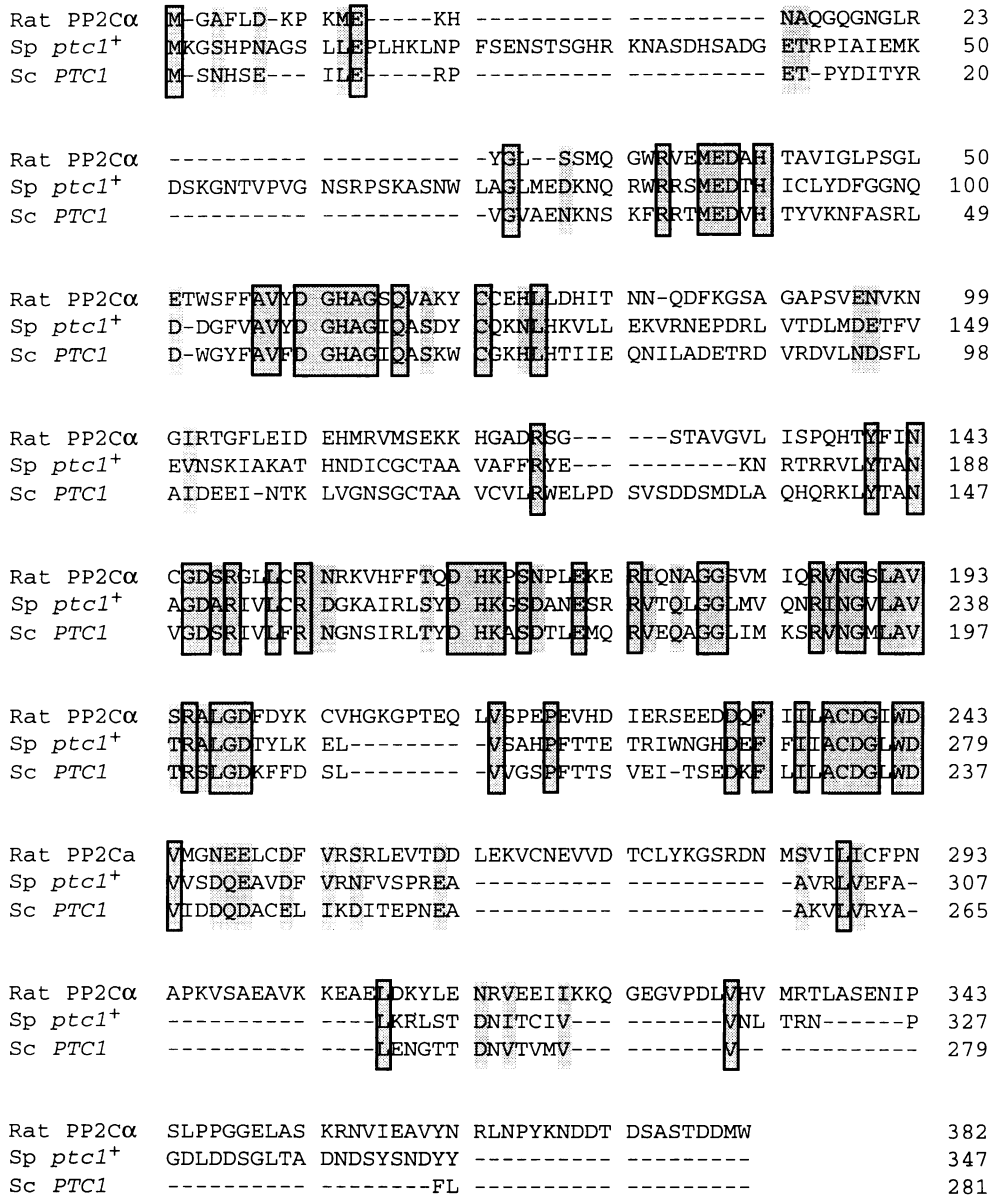


FIG. 3. Comparison of the predicted amino acid sequence of *ptc1*$^+$ with rat PP2Cα (35) and *S. cerevisiae PTC1* (18). Conserved amino acid residues are shaded, and residues identical in the three sequences are boxed.

mutant *swo1-26* Δ*ptc1* could grow at 25°C, and the phenotype at 35.5°C is very similar to that of the *swo1-26* mutant strain (data not shown).

***ptc1*$^+$ mRNA levels increase during heat shock.** The fact that *ptc1*$^+$ was isolated as a multicopy suppressor of hsp90 mutation implied the involvement of this gene in the heat shock response, though Δ*ptc1* cells grew normally even at 36°C. We examined whether heat shock to the cell altered the expression of *ptc1*$^+$. Wild-type *S. pombe* cells exponentially growing in YES medium at 25°C were shifted to 35°C, and aliquots were harvested after 5, 10, 20, 40, and 60 min for Northern blot analysis (Fig. 6). Transcripts of *ptc1*$^+$ were detected as ~2.4-kb mRNA. The level of *ptc1*$^+$ mRNA rapidly increased after the temperature shift, peaking at a 5- to 10-fold-greater level at 20 min. The level of *ptc1*$^+$ mRNA then gradually decreased through the 40- and 60-min samples. The same membrane was subjected to hybridization with a control probe, the *leu1*$^+$ gene (lower panel). The amount of *leu1* transcript showed little fluctuation during the experiment.

**A *ptc1* mutant is sensitive to heat shock.** We next analyzed the ability of the Δ*ptc1* mutant to survive exposure to extreme temperatures. *S. pombe* cells growing exponentially are killed by a short incubation at 47.5°C (5). However, if the cells are pretreated by a mild heat shock, a majority of them can adapt and survive this lethal temperature (6). In the experiments whose results are shown in Fig. 7, wild-type and *ptc1* deletion mutant cells were grown at 25°C to mid-log phase in YES medium. The cultures were shifted to 48°C at time zero either with or without preincubation at 37°C for 30 min. Following heat treatment, cells were transferred to ice and plated to assay the colony-forming ability. When the cultures were shifted directly from 25 to 48°C (Fig. 7A), Δ*ptc1* cells died much more

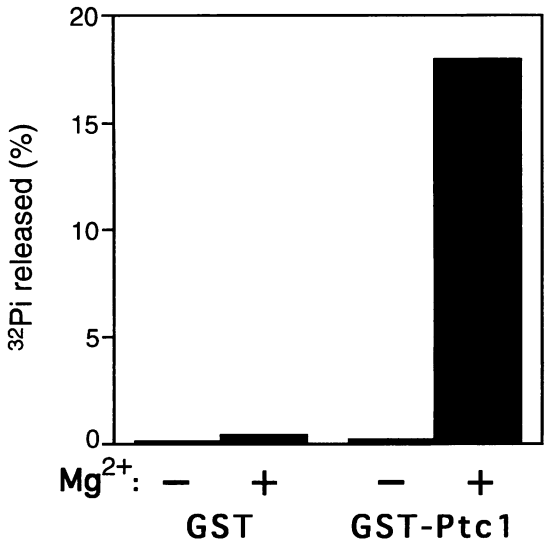


FIG. 4. Casein phosphatase activity of Ptc1 protein produced in *E. coli*. Using GSH-Sepharose beads, GST and GST-Ptc1 fusion protein were purified from the cell lysates of *E. coli* DH5α strains transformed with either a vector, pGEX-KG, or pGEX-KG-ptc1 plasmid. After extensive washing, the protein-GSH-Sepharose bead complexes were incubated with $^{32}$P-labeled casein in the absence (−) or presence (+) of 20 mM $MgCl_2$. The activity is shown by the amount of released $^{32}$P as a percentage of $^{32}$P-casein added in the reaction mixture.

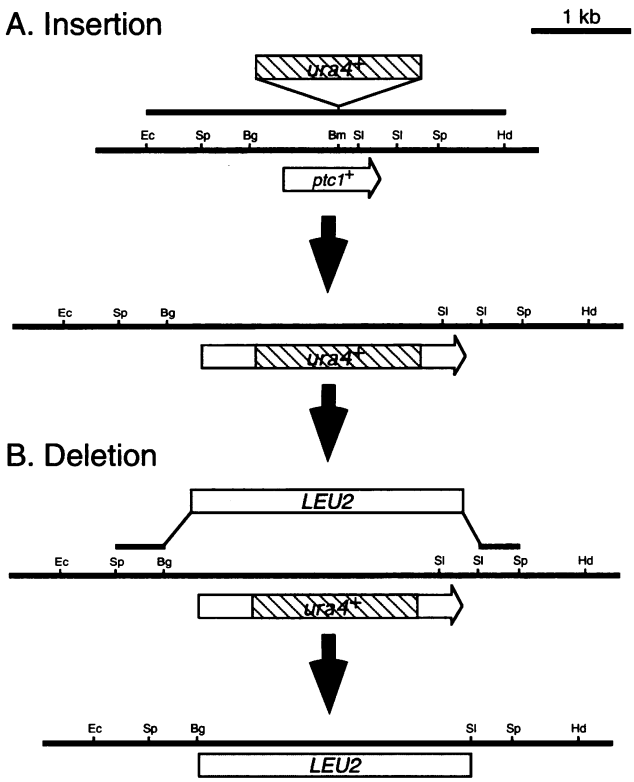


FIG. 5. Gene disruption of *ptc1*$^+$. (A) Insertion of *ura4*$^+$ gene. The *Eco*RI-*Hin*dIII fragment containing the *ptc1*$^+$ gene was cleaved at the *Bam*HI site and the marker *ura4*$^+$ gene was inserted. This fragment was used to transform a diploid *S. pombe* strain (*h*$^+$/*h*$^+$ *leu1/leu1 ura4/ura4*) to replace a chromosomal *ptc1*$^+$ locus with the disrupted gene by homologous recombination. The transformants were subjected to sporulation, and haploid *ptc1* disruptants (Ura$^+$ Leu$^-$) were recovered. (B) Deletion of the *ptc1*$^+$ ORF. The *Sph*I fragment of *ptc1*$^+$ was digested by *Bgl*II and *Sal*I to substitute the ORF with the *S. cerevisiae LEU2* gene fragment. A haploid strain (construction shown in panel A, containing a *ura4*$^+$-inserted *ptc1* gene) was used for transformation, and Ura$^-$ Leu$^+$ transformants were selected. Complete deletion of *ptc1* ORF was confirmed by Southern hybridization (data not shown). Bg, *Bgl*II; Bm, *Bam*HI; Ec, *Eco*RI; Hd, *Hin*dIII; Sl, *Sal*I; Sp, *Sph*I.

rapidly than wild-type cells. After 15 min of incubation at 48°C, only 0.3% of the Δ*ptc1* cells survived, whereas 20% of the *ptc1*$^+$ cells were viable. However, both wild-type and *ptc1* disruptants acquired the same level of thermotolerance after pretreatment at 37°C (Fig. 7B). Hence, *ptc1* mutant cells are sensitive to heat shock, but *ptc1*$^+$ is not required for thermoresistance induced by prior treatment at 37°C.

**Ptc1 contributes a minor portion of total PP2C activity.** In order to characterize PP2C activity in wild-type and *ptc1* mutant cells, we fractionated cell lysates by gel filtration or ion-exchange chromatography and assayed PP2C activity. Wild-type *S. pombe* cells were grown in YES medium to mid-log phase. The cell lysate was prepared by disrupting the cell wall with glass beads and was then applied to a Superose 12 column or a MonoQ column. Bovine casein was $^{32}$P labeled by the catalytic subunit of cyclic AMP-dependent protein kinase and used as a substrate. Since both PP2A and PP2C have casein phosphatase activity, two different reaction conditions were used to distinguish those activities (3). PP2A activity does not require divalent cation and can be assayed in the presence of 1 mM EDTA. To measure PP2C activity, which is dependent on $Mg^{2+}$, 20 mM $MgCl_2$ was added instead of EDTA, and 100 nM okadaic acid was employed to inhibit PP2A activity.

As shown in Fig. 8, gel filtration chromatography identified $Mg^{2+}$-dependent, okadaic acid-resistant activity (PP2C) as a single peak that eluted at the same volume as ovalbumin, a 43-kDa marker protein. The calculated molecular mass of Ptc1 is 40 kDa. This apparent molecular mass is similar to that of mammalian PP2C enzymes which are known to exist as monomeric proteins of about 43 kDa (22). On the other hand, the assay with EDTA detected two peaks, corresponding to about 150 and 40 kDa. The first peak might correspond to the *S. pombe* equivalent of the mammalian PP2A holoenzyme, which is a heterotrimer of A, B, and C subunits with a molecular mass of around 150 kDa (2). The second peak is close to the expected size of the monomeric Ppa1 and Ppa2 proteins, catalytic subunits of PP2A in *S. pombe* (35.3 and 36.5 kDa, respectively) (14).

By MonoQ chromatography, PP2C-like activity was separated into two broad peaks, eluting at 0.26 and 0.35 M NaCl (Fig. 9A). Both of the peaks were resistant to 2 μM okadaic acid and completely dependent on the addition of $Mg^{2+}$ for activity (data not shown). Furthermore, under these assay conditions no decrease in the activity was detected in the extract from the strain lacking *ppa2*$^+$ gene (data not shown), which encodes a major PP2A activity in *S. pombe* (14). Hence, the activity measured under this condition did not contain PP2A activity. These results indicate that there are at least two different forms of PP2C in *S. pombe*. Consistent with this, the peak fraction of PP2C activity in gel filtration chromatography (fraction 13 in Fig. 8) was separated into two peaks by a MonoQ column (data not shown).

The MonoQ profile of PP2C activity in a cell extract from a *ptc1* deletion strain differed slightly from that of the wild type (Fig. 9A). There was a small decrease (~10%) in the first peak that was reproduced in four of four experiments. We examined

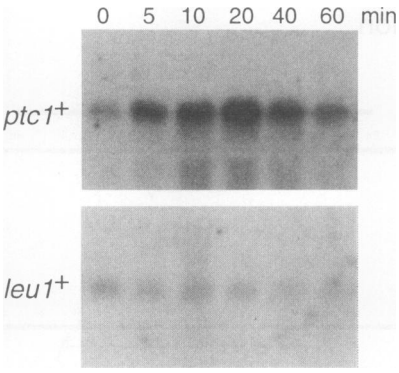


FIG. 6. Induction of *ptc1*$^+$ mRNA by heat shock. Wild-type *S. pombe* cells exponentially growing in YES medium at 25°C were shifted to 35°C, and aliquots were harvested after 5, 10, 20, 40, and 60 min for Northern blot analysis with the 1.1-kb *ptc1*$^+$ ORF fragment as a probe. A severalfold increase of *ptc1*$^+$ mRNA was detected at 20 min after heat shock. The same membrane was also hybridized with a control *leu1*$^+$ probe. The amount of *leu1* mRNA showed little fluctuation during the heat shock treatment.

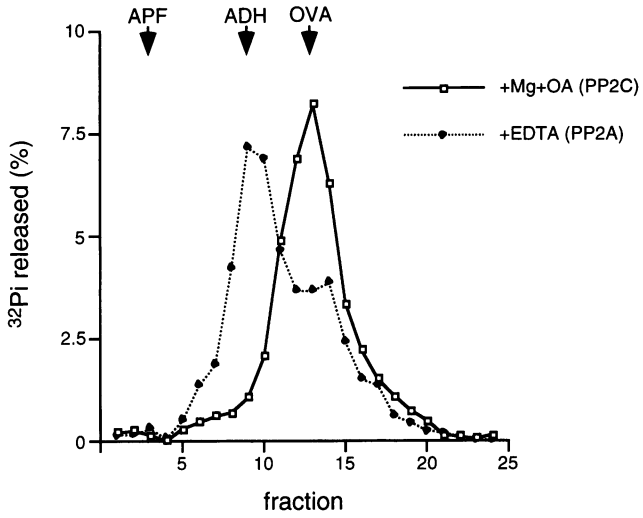


FIG. 8. Gel filtration chromatography of casein phosphatase activity in *S. pombe* crude extract. Wild-type cell extract was chromatographed on a Superose12 HR10/30 column, and the casein phosphatase activity of PP2C enzymes was assayed by incubation in the presence of 20 mM $MgCl_2$ and 100 nM okadaic acid [+Mg+OA (PP2C)]. The PP2A activity was measured separately, using the reaction buffer containing 1 mM EDTA [+EDTA (PP2A)]. The elution volume of molecular mass standard proteins are shown by arrows. APF, apoferritin (450 kDa); ADH, alcohol dehydrogenase (150 kDa); OVA, ovalbumin (40 kDa).

the MonoQ profile of PP2C activity in a cell extract from a *ptc1* overproducer strain in order to confirm whether Ptc1 contributed to the PP2C activity of the first peak. The first peak of PP2C activity increased dramatically (>10-fold) in cells expressing *ptc1*$^+$ from the strong *nmt1* promoter (Fig. 9B). These data indicate that the PP2C activity in the first peak is derived from the protein products of at least two genes, of which *ptc1*$^+$ contributes only a small portion.

Since *ptc1*$^+$ mRNA levels increase 5- to 10-fold during heat shock, it might be expected that differences in PP2C activity

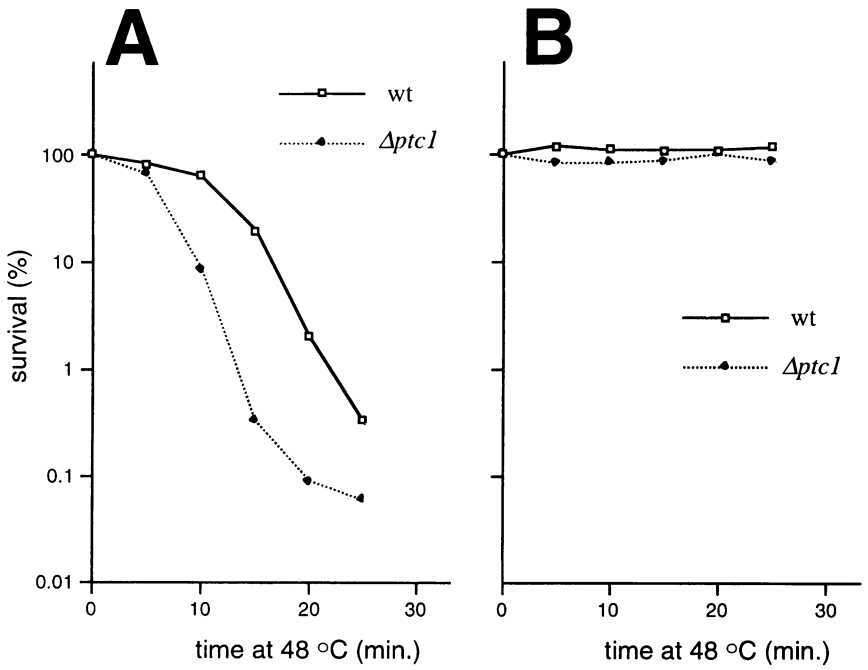


FIG. 7. Thermosensitivity of *ptc1* disruptant. Wild-type (*h*$^-$ *leu1 ura4*) and *ptc1* deletion mutant (*h*$^-$ *leu1 ura4 ptc1*::*LEU2*) cells were grown to mid-log phase at 25°C and then shifted up to 48°C without (A) or with (B) pretreatment at 37°C for 30 min. After 5, 10, 15, 20, and 25 min aliquots were taken and assayed for their colony-forming abilities on the plates at 25°C. The cells lacking Ptc1 die more rapidly than do the wild-type cells when exposed to 48°C, though a mild heat shock can induce thermotolerance in both strains. wt, wild type.

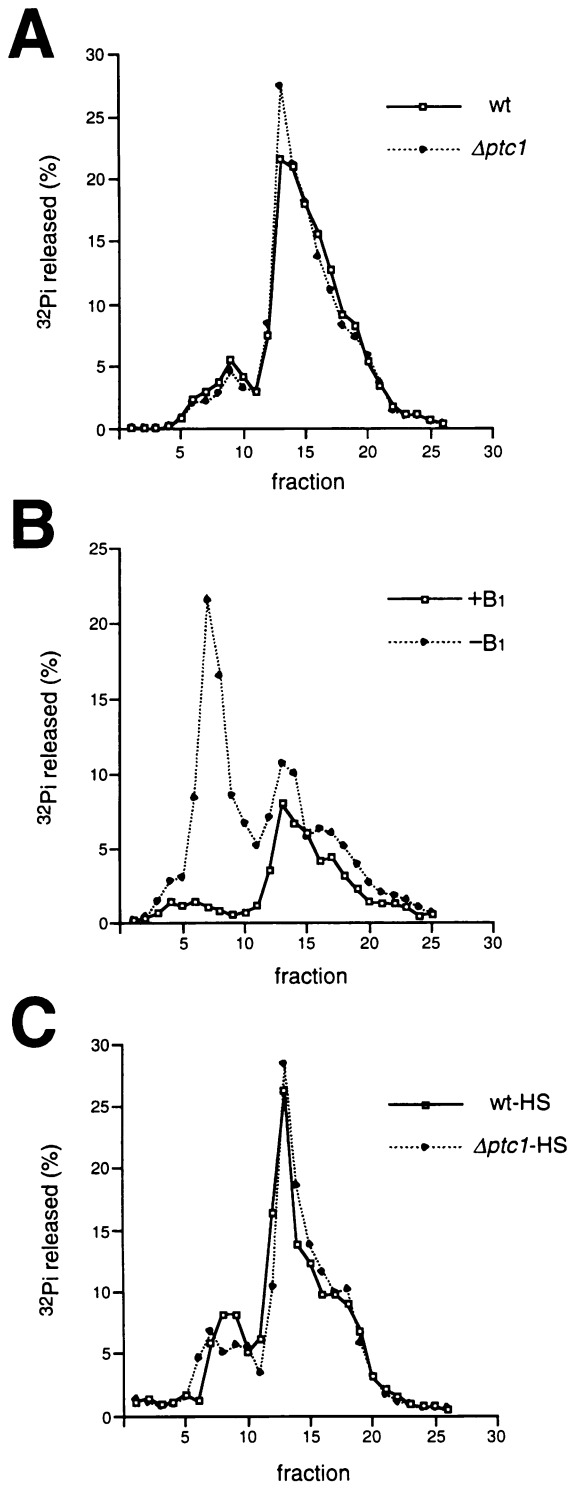


FIG. 9. MonoQ chromatography of PP2C activity in cell extracts. Extracts were chromatographed through a MonoQ HR5/5 column with a 0.1 to 1.0 M NaCl gradient. PP2C activity of each fraction was measured in the presence of 20 mM $MgCl_2$ and 100 nM okadaic acid with $^{32}P$-labeled casein as a substrate. (A) Wild-type (wt) and *ptc1* deletion mutant (Δ*ptc1*) extracts. (B) Wild-type *S. pombe* cells transformed with the pREP1-ptc1Ha6H plasmid were grown in the presence ($+B_1$) or absence ($-B_1$) of thiamine for 18 h to induce the expression of $ptc1^+$ under the regulation of the $nmt1^+$ promoter. Note that in this experiment fractions were diluted twofold to ensure that the assay was maintained in the linear range. (C) Thirty minutes prior to harvesting, wild-type (wt-HS) and *ptc1* deletion mutant (Δ*ptc1*-HS) cells were shifted from 25 to 35°C.

between the wild type and Δ*ptc1* would be accentuated in heat-shocked cells. Consistent with this prediction, in cultures harvested 30 min after a shift from 25 to 35°C, we found that PP2C activity in the first MonoQ peak was significantly higher (~50%) in $ptc1^+$ cells compared with Δ*ptc1* cells (Fig. 9C). These data are consistent with Ptc1 having a specialized role in the heat shock response.

## DISCUSSION

In this report we have described the cloning and analysis of the fission yeast $ptc1^+$ gene, encoding a PP2C homolog. Ptc1 protein expressed in *E. coli* or in *S. pombe* has a serine/threonine protein phosphatase activity that is dependent on $Mg^{2+}$ and resistant to okadaic acid. Thus, Ptc1 has two of the biochemical properties that most clearly distinguish PP2C enzymes from other types of serine/threonine protein phosphatases. The molecular mass of Ptc1, ~40 kDa, is also typical of mammalian PP2C enzymes. Remarkably, the amino acid sequence of Ptc1 is only ~20% identical to that of mammalian PP2C. This value is perhaps unexpectedly low, considering that catalytic subunits of fission yeast PP1 (29) and PP2A enzymes (14) are 70 to 80% identical to those of the rabbit enzymes. It has been suggested that PP1 and PP2A enzymes are highly conserved because they have multiple roles and interact with many proteins. The divergence of PP2C enzymes suggests that they might have limited cellular roles and function predominantly as monomer enzymes.

Divergence of PP2C enzymes would be accelerated if distinct cellular functions were carried out by specialized subtypes. In fact, three key results presented in this paper strongly indicate that Ptc1 has a unique function in the heat shock response. First, $ptc1^+$ was isolated as a multicopy suppressor of a mutation in a gene encoding an hsp90 homolog. Second, the level of $ptc1^+$ mRNA increases dramatically during heat shock. Third, *ptc1* deletion mutant cells are 10- to 50-fold more sensitive to killing by exposure to high temperatures.

Ptc1 phosphatase does not seem to be important for unstressed cells. A *ptc1* deletion mutant has a normal growth rate, and it conjugates and sporulates normally (data not shown). Consistent with this, biochemical analyses showed that Ptc1 protein contributes only a minor portion of the total PP2C activity in fission yeast cells, at least as measured by using casein as the substrate. A slight decrease in one peak of PP2C activity identified in MonoQ chromatography of extracts from *ptc1* deletion mutant cells is accentuated by heat shock. In mammalian cells, two PP2C isozymes (PP2Cα and PP2Cβ [21, 22]) have been reported. We have recently cloned two additional PP2C homologs, $ptc2^+$ and $ptc3^+$, by PCR with *S. pombe* genomic DNA as a template (33). The $ptc2^+$ gene does not seem to be involved in cellular response to heat shock; overproduction of Ptc2 cannot rescue the temperature-sensitive growth defect of *swo1-26*, and unlike $ptc1^+$, $ptc2^+$ mRNA is not heat shock inducible, nor are Δ*ptc2* cells sensitive to exposure to extreme temperatures. These results suggest that $ptc1^+$ and $ptc2^+$ have different cellular roles and/or substrate specificities. Considering the observation that PP2C enzymes exist as monomeric proteins both in mammalian cells (22) and in yeasts, the PP2C family might be composed of a number of

catalytic subunits, each of which has a different substrate specificity.

One gene encoding PP2C in the budding yeast *S. cerevisiae* has been described (18). Mutation of budding yeast *PTC1* was found to cause a severe growth defect in mutants lacking the *PTP2* protein tyrosine phosphatase gene. Budding yeast cells having a *ptc1* deletion grew normally at 30°C but grew very poorly at 37°C. Interestingly, the level of *PTP2* mRNA is significantly increased in cells examined 30 min following a temperature shift from 30 to 39°C. Cells having a deletion of *PTP2* were also more sensitive to an 18-h incubation at 39°C (30). These results suggest that budding yeast *PTC1* and *PTP2* phosphatases are important for long-term growth at elevated temperatures and perhaps also have a role in the heat shock response. In higher eukaryotes, a protein tyrosine phosphatase, CL100, is induced by heat shock or oxidative stress (13). These observations suggest that protein dephosphorylation carried out by tyrosine and serine/threonine phosphatases plays important roles in the response or recovery from heat shock and perhaps other types of environmental stress. Our data indicate that PP2C activity is very important in the heat shock response. The genetic interaction between $ptc1^+$ and *swo1-26* might be explained in any number of ways, but one obvious possibility is that hsp90 might be one of the substrates of Ptc1. hsp90 is phosphorylated by casein kinase II (7, 16, 27). Legagneux et al. (17) reported the increased turnover of hsp90 phosphate groups in HeLa cells submitted to heat shock, which could be explained by an increased hsp90 phosphatase activity. In vitro experiments to measure the activity of Ptc1 against hsp90 and other phosphoproteins using bacterially produced and highly purified Ptc1 protein are now feasible.

The studies described in this report are among the first demonstrating a physiological defect caused by loss of PP2C activity. They also are among the first showing that a specific protein phosphatase has an important role in the process by which cells attempt to survive heat shock. These studies provide the framework for undertaking further genetic and biochemical analyses aimed at defining the functional roles of Ptc1 in the stress response. Moreover, we are confident that the cloning of $ptc1^+$ supplies the information needed to access all genes encoding PP2C enzymes in *S. pombe*. The cloning of these genes will provide the tools necessary to achieve a comprehensive understanding of the cellular processes regulated by PP2C enzymes.

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