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Mutations in a Protein Tyrosine Phosphatase Gene (*PTP2*) and a Protein Serine/Threonine Phosphatase Gene (*PTC1*) Cause a Synthetic Growth Defect in *Saccharomyces cerevisiae*

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Two protein tyrosine phosphatase genes, *PTP1* and *PTP2*, are known in *Saccharomyces cerevisiae*. However, the functions of these tyrosine phosphatases are unknown, because mutations in either or both phosphatase genes have no clear phenotypic effects. In this report, we demonstrate that although *ptp2* has no obvious phenotype by itself, it has a profound effect on cell growth when combined with mutations in a novel protein phosphatase gene. Using a colony color sectoring assay, we isolated 25 mutants in which the expression of *PTP1* or *PTP2* is required for growth. Complementation tests of the mutants showed that they have a mutation in one of three genes. Cloning and sequence determination of one of these gene, *PTC1*, indicated that it encodes a homolog of the mammalian protein serine/threonine phosphatase 2C (PP2C). The amino acid sequence of the *PTC1* product is ~35% identical to PP2C. Disruption of *PTC1* indicated that the *PTC1* function is nonessential. In contrast, *ptc1 ptp2* double mutants showed a marked growth defect. To examine whether *PTC1* encodes an active protein phosphatase, a glutathione *S*-transferase (GST)–PTC1 fusion gene was constructed and expressed in *Escherichia coli*. Purified GST-PTC1 fusion protein hydrolyzed a serine phosphorylated substrate in the presence of the divalent cation Mg^{2+} or Mn^{2+}. GST-PTC1 also had weak (~0.5% of its serine phosphatase activity) protein tyrosine phosphatase activity.

Reversible protein phosphorylation is a key mechanism to modulate the activity of proteins. It plays a central role in a variety of types of cellular regulation, including the control of metabolism, cell cycle, cell proliferation, and differentiation. In particular, phosphorylation of tyrosine residues is a crucial step in a number of eukaryotic signal-transducing pathways. Because the extent of tyrosine phosphorylation is affected by the activities of both protein tyrosine kinases and protein tyrosine phosphatases (PTPases), a comprehensive understanding of protein tyrosine phosphorylation in signal transduction must take both enzymes into consideration. While it is possible that PTPases affect the extent of phosphorylation by indiscriminately removing phosphates from phosphotyrosine residues, the recent discovery of a large and diverse PTPase gene family suggests more specific roles (5, 15, 50).

The PTPase gene family is composed of two major subdivisions: transmembrane (or receptor-linked) PTPases and cytosolic (or nonreceptor) PTPases. Transmembrane PTPases are composed of an extracellular receptor domain, a transmembrane segment, and usually two highly conserved PTPase domains of ~300 amino acids (aa) in size (30, 31, 59). In contrast, all cytosolic PTPases so far identified have only one conserved PTPase domain (5). Typical cytosolic PTPases also have accessory domains of varied structural motifs, at either the N- or C-terminal end of the catalytic domain. Some accessory domains are related to well-characterized protein families. For example, several cytosolic PTPases have two SH2 domains (14, 43, 44, 54, 62), whereas PTP-H1 and PTP-MEG have a domain homologous to the band 4.1 protein (18, 64). The roles of the accessory domains are not yet well understood, although it is generally believed that they mediate the subcellular localization of the cytosolic PTPases.

Despite the plethora of cytosolic PTPases, their functions are not obvious. This difficulty is due partly to the paucity of systems in which the modulation of a PTPase activity is reflected in the physiological changes of cells. In this regard, the yeast *Saccharomyces cerevisiae* is an ideal organism for study of the role of cytosolic PTPases in signal transduction. Enzymes that are involved in protein phosphorylation, such as protein kinase C, cyclic AMP (cAMP)-dependent protein kinase, mitogen-activated protein (MAP) kinase, and type 1, 2A, and 2B serine/threonine phosphatases, are very similar between mammalian cells and yeast cells. Thus, it can be expected that the basic mechanisms of the regulation of cell function by protein phosphorylation, including the role of PTPases, are similar in yeast and mammalian cells.

Several genes homologous to mammalian PTPase genes have been identified in both fission and budding yeasts: *pyp1*, *pyp2*, and *pyp3* from *Schizosaccharomyces pombe* (36, 37, 41, 42) and *PTP1* and *PTP2* from *S. cerevisiae* (19, 20, 25, 40). These yeast PTPases are all cytosolic enzymes with significant sequence similarity to the mammalian PTPases. In the case of *S. pombe*, all three genes are implicated in mitotic control (36, 37, 42). In contrast, the *S. cerevisiae PTP1* and *PTP2* genes have no known function. The disruption of *PTP1* and *PTP2*, either individually or together, had no clear phenotype (19, 20, 25). Structurally, PTP1 (335 aa) is composed of little more than the catalytic domain itself, whereas PTP2 (750 aa) has an extended

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TABLE 1. Yeast strains used in this study

Strain	Genotype	Source
CH1035	*MATa ade2 ade3 leu2 ura3 lys2 can1*	J. Kranz and C. Holm
CH1462	*MATα ade2 ade3 leu2 ura3 his3 can1*	J. Kranz and C. Holm
TM44	*MATa ade2 ade3 leu2 ura3 lys2 can1 ptp1::hisG ptp2::hisG*	This study
TM45	*MATα ade2 ade3 leu2 ura3 his3 can1 ptp1::hisG ptp2::hisG*	This study
TM44P1	TM44 + pSSP15	This study
TM45P1	TM45 + pSSP15	This study
TM44P2	TM44 + pSSP25	This study
TM45P2	TM45 + pSSP25	This study
FY23	*MATa leu2 ura3 trp1*	F. Winston
FY86	*MATα leu2 ura3 his3*	F. Winston
TM102	*MATa/MATα leu2/leu2 ura3/ura3 TRP1/trp1 his3/HIS3*	FY23 × FY86
TM103	*MATa leu2 ura3 trp1 ptp1::LEU2*	FY23
TM107	*MATa leu2 ura3 trp1 ptp2::LEU2*	FY23
TM111	*MATa leu2 ura3 trp1 mih1::LEU2*	FY23
TM116	*MATa/MATα leu2/leu2 ura3/ura3 TRP1/trp1 his3/HIS3 PTC1/ptc1::URA3*	This study
TM126	*MATα leu2 ura3 his3 ptc1::URA3*	Segregant from TM116
TM165	*MATa leu2 ura3 trp1 ptp1::hisG ptp2::hisG ptc1::LEU2* + pSSP25	This study
TM166	*MATα leu2 ura3 his3 ptp1::hisG ptp2::hisG ptc1::LEU2* + pSSP25	This study

N-terminal accessory domain that is not homologous to any known protein. The catalytic domains of PTP1 and PTP2 are about 30% identical to those of mammalian cytosolic PTPases. Recently, another *S. cerevisiae* tyrosine phosphatase, YVH1, which is remotely related to other PTPases, was identified (21). However, the function of *YVH1* seems independent of that of *PTP1* or *PTP2*.

Considering the crucial roles that protein tyrosine phosphorylation plays in regulating cell proliferation and differentiation in higher eukaryotic cells, the lack of phenotypes in *ptp1 ptp2* double-disrupted yeast strains was unexpected. There are several possible explanations for this lack of phenotype. For example, the participation of these PTPase genes might be crucial under conditions more stressful than the favorable laboratory culture conditions. Another plausible explanation is that there may be redundancy in the signal transduction pathways in which these PTPases play a role. There are numerous cases of functionally redundant isozymes in *S. cerevisiae*, including several serine/threonine phosphatases (13, 46, 57). However, attempts to isolate additional PTPase genes by cross-hybridization to *PTP1* or *PTP2* have not been successful. Alternatively, there might be functionally redundant branches in a signal transducing pathway, one of which involves the *PTP1* and/or *PTP2* phosphatases; another branch may or may not involve any PTPase. In either case, it will be possible to determine the function of the PTPases by isolating mutants that are impaired in the redundant gene or pathway.

A synthetic lethal screen is a method to identify such redundant genes or pathways (3, 12, 16, 40). Two mutations are defined as synthetic lethal when each is nonlethal yet the combination of them causes lethality. Utilizing a synthetic lethal screen that makes use of a colony color sectoring assay (23, 26, 27), we isolated mutants that require the *PTP1* or *PTP2* gene for growth. The results indicated that the yeast PTPases and a novel serine/threonine phosphatase play an important role in the same signal transduction pathway.

MATERIALS AND METHODS

Strains, media, and general methods. All yeast strains used in this study are listed in Table 1. Because CH1035 and CH1462 have mixed strain backgrounds (27), their derivatives (TM44 and TM45) were used solely for the purpose of mutant isolation. Further analyses were performed by using isogenic strains constructed on the background of S288C (38).

Growth media and standard yeast manipulations were as described by Rose et al. (47). SC-Leu and SC-Ura stand for SC leucine dropout and SC uracil dropout media, respectively (47). YEPGal and SGal media are identical to YEPD and SD media, respectively, except that 2% galactose is substituted for glucose as the carbon source. SGal+Ade, Leu,Ura is SGal supplemented with adenine sulfate, leucine, and uracil at the concentrations specified by Rose et al. (47). Yeast cells were transformed by the lithium acetate method (24) with single-stranded DNA as the carrier (17, 53).

The following *Escherichia coli* strains were used: DH5 and DH5α for the propagation of plasmids; XL1-Blue for the preparation of single-stranded DNA by using VCSM13 helper phage; and NM522 for expression of the glutathione *S*-transferase (GST)-PTC1 fusion protein. Other recombinant DNA procedures were carried out as described by Sambrook et al. (51).

Construction of parental yeast strains for synthetic lethal screening. Both *PTP1* and *PTP2* genes were disrupted in the haploid strains CH1305 and CH1462 (27) to produce strains TM44 and TM45, respectively. To facilitate future genetic manipulation, it was necessary to make multiple disruptions without altering the auxotrophic markers of the strains. Thus, both *PTP1* and *PTP2* were disrupted by using a marker cassette that has a *URA3* gene between duplicated copies of a *Salmonella hisG* gene segment (1). After disruption of a gene with the *hisG-URA3-hisG* cassette, *URA3* can be removed by homologous recombination between the two *hisG* sequences to leave a single *hisG* copy inserted in the gene. The excision of *URA3* was selected for by 5-fluoroorotic acid (4). Because the resulting disruptant strains are *ura3* auxotrophs, multiple disruption is possible by repeating the same process with other genes. The structures of the disrupted genes were confirmed by Southern analysis. For the *PTP1* disruption, the *Hin*dIII-*Eco*RI fragment of the *PTP1* gene in which most of *PTP1* open reading frame (*Nco*I-*Nsi*I) was replaced by the *hisG-URA3-hisG* fragment was used for transformation. For the *PTP2* disruption, the *Bam*HI-*Sau*3AI fragment of the *PTP2* gene in which most of *PTP2* open reading frame (*Hpa*I-*Pst*I) was replaced by the *hisG-URA3-hisG* fragment was used. TM44 and TM45 were transformed with pSSP15 to give rise to TM44P1 and

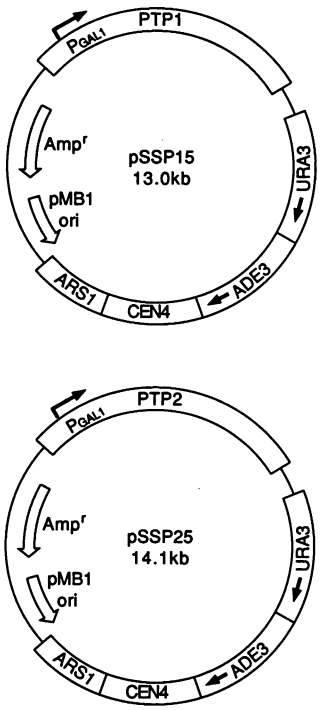

FIG. 1. Schematic representations of the screening plasmids used in this work. The promoter fusion gene, P_{GAL1}-*PTP1*, was constructed by ligating the yeast *GAL1* promoter (65) to a 1.6-kb *Ssp*I-*Cla*I fragment that corresponds to nucleotide positions 660 through 2218 of the published *PTP1* sequence (20). Similarly, P_{GAL1}-*PTP2* was constructed by fusing the *GAL1* promoter and a 2.7-kb *Avr*II-*Sau*3AI fragment that corresponds to nucleotide positions 948 through 3608 of the published *PTP2* sequence (19). The *GAL1* fusion genes are individually inserted into the parental plasmid pCH1122 (27). Ori, origin of replication.

TM45P1 and with pSSP25 to give rise to the TM44P2 and TM45P2, respectively (Table 1).

Plasmids and libraries. pCH1122 (a YCp50 derivative that carries the *ADE3* gene) was provided by J. Kranz and C. Holm (Harvard University) (27). The screening plasmid pSSP15 was constructed by inserting the *GAL1* promoter (P_{GAL1})-*PTP1* fusion gene between the *Sal*I and *Nru*I sites of pCH1122. Another screening plasmid, pSSP25, was similarly constructed by using the P_{GAL1}-*PTP2* fusion gene (Fig. 1).

The pGEX2T GST gene fusion vector was obtained from Pharmacia. To construct pGEX2T-PTC1, which expresses GST-PTC1 fusion protein, the initiation Met codon (ATG) of the *PTC1* gene was replaced with a sequence (GAATTCGC) that contains an *Eco*RI site and lacks a Met codon. The structure of the modified gene was verified by nucleotide sequencing. The 1.0-kb *Eco*RI-*Hpa*I fragment of the modified *PTC1* gene was inserted at the unique *Eco*RI site of pGEX2T, fusing the *PTC1* coding region in frame with the GST domain.

A yeast genomic library constructed in YEp13 (32) was a gift from E. Elion (Harvard Medical School).

Isolation of mutants that require the *PTP1* or *PTP2* gene for growth. Synthetic lethal screening was done with a pair of yeast strains with different mating types and nutritional requirements. The isolation of mutants from two parental strains that can be mated facilitates later analyses by a complementation test. For the *PTP1*-dependent mutant screen, TM44P1 (*MAT***a** *lys2* + pSSP15) and TM45P1 (*MAT*α *his3* + pSSP15) were used as the parental strains; for the *PTP2*-dependent mutant screen, TM44P2 (*MAT***a** *lys2* + pSSP25) and TM45P2 (*MAT*α *his3* + pSSP25) were used.

Mutagenesis was performed with UV irradiation as follows. Parental strains were grown overnight in SC-Ura medium. The cell density was determined by measuring the optical density at 600 nm (OD_{600}), and the culture was diluted appropriately in water. After plating onto YEPGal plates, cells were UV irradiated at 80 to 120 J/m^2, using a UV cross-linker (Stratalinker; Stratagene). These UV doses resulted in about 5 to 20% survival. Irradiated cells were incubated at 30°C in the dark for about 8 days until the red color fully developed. Colonies without sectors were retested twice for nonsectoring colony morphology. Strains that reproducibly showed the nonsectoring red colony phenotype were retained for further analysis. Because expression of the *PTP* gene on plasmid pSSP15 or pSSP25 is galactose inducible, growth of *PTP*-dependent mutants is expected to be galactose dependent. To test whether any nonsectoring mutants were galactose dependent, colonies were replica plated onto YEPD and YEPGal plates and incubated overnight.

To isolate *PTP1*-dependent mutants, approximately 4.0×10^4 mutagenized colonies of TM44P1 or TM45P1 were screened. Of 29 nonsectoring red colonies thus isolated, only three showed galactose-dependent growth. For the *PTP2*-dependent mutants, approximately 3.8×10^4 mutagenized colonies of TM44P2 and TM45P2 were screened to obtain 47 nonsectoring red colonies. Of these, 22 mutants were galactose dependent. Only those strains that were both nonsectoring and galactose dependent were further analyzed.

Dominance-recessiveness and complementation tests of *PTP*-dependent mutants. To test the dominance or recessiveness of the *PTP*-dependent mutations, each *PTP*-dependent mutant was crossed with a parental strain of the opposite mating type that harbors the same screening plasmids; i.e., the TM44P1-derived mutants were mated with TM45P1, and the TM45P2-derived mutants were mated with TM44P2. Crossing was performed by mixing two strains on YEPGal plates and incubating them at 30°C for 2 days. Diploid cells (Lys^+ His^+) were selectively grown by replica plating the mating mixture onto SGal+Ade,Leu,Ura plates. The resulting diploid cells were again replica plated onto YEPD and YEPGal plates to evaluate the galactose dependence of their growth. Diploid strains were also tested for the colony sectoring phenotype on YEPGal plates.

For the complementation test, *PTP2*-dependent mutants of opposite mating types were crossed pairwise, and the resulting diploid strains were tested for galactose dependence and the colony sectoring phenotype as described above.

Cloning and sequence determination of the *PTC1* gene. The *PTC1* gene was cloned by complementation of galactose-dependent cell growth. Strain P1d5215 was transformed with a YEp13 yeast genomic library. Transformed cells were plated on SC-Leu plates and selected for galactose-independent cell growth. Three galactose-independent colonies were identified among approximately 10,000 Leu^+ transformants (estimated from the number of transformants on SGal-Leu plates). The YEp13-based plasmids were rescued from these three transformants into *E. coli* DH5 by a standard method (47). Similarly, one plasmid was isolated by using the P2d5314 mutant strain.

The 2.6-kb *Hin*dIII fragment from pPD111 was cloned into

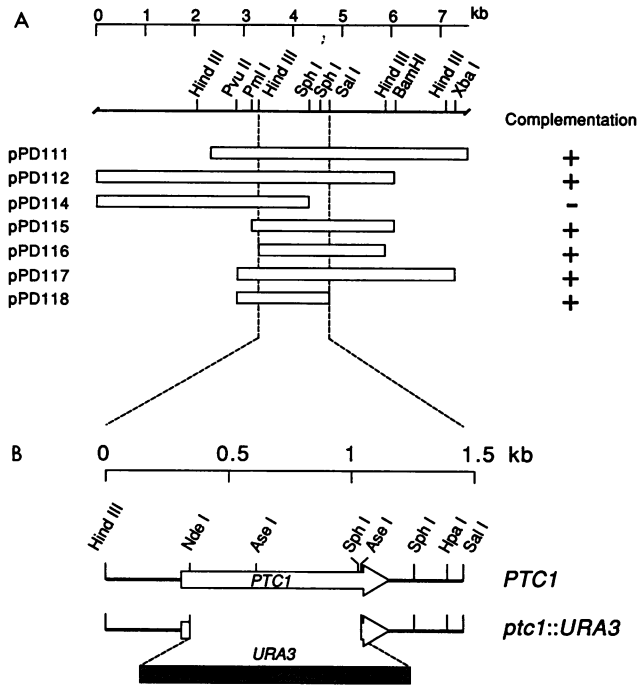

FIG. 2. (A) Restriction map of the *PTC1* region. The open bars under the restriction map indicate genomic DNA clones characterized. pPD111 and pPD112 were isolated from a yeast genomic DNA library by complementing the *ptc1* mutations. The other DNA clones were constructed from various restriction fragments of pPD111 or pPD112. The results of the complementation test for the *ptc1* mutations are shown on the right. (B) Enlargement of the shortest complementing region (1.5-kb *Hin*dIII-*Sal*I segment). The large arrow indicates the *PTC1* coding region. The structure of the *ptc1*::*URA3* disrupted gene that was used for construction of the *ptc1* null mutants is also shown.

pRS315 (55) in both orientations, and nested deletions were created by the method of Henikoff (22). The nucleotide sequence of the 1.5-kb *Hin*dIII-*Sal*I fragment was determined in both orientations by the chain termination method, using a modified T7 RNA polymerase and single-stranded DNA templates (52, 60).

Disruption of *PCT1*. Gene disruption was done by the method of Rothstein (48). The *PTC1* gene was disrupted by replacing the 0.7-kb *Nde*I-*Ase*I segment of the *PTC1* gene with the 1.1-kb *Hin*dIII fragment of *URA3* (Fig. 2). The 1.9-kb *Hin*dIII-*Sal*I fragment that contains the *ptc1*::*URA3* disrupted gene was used for integrative transformation of the diploid strain TM102 to give rise to the heterozygous disruptant strain TM116. Ura^+ transformants were selected, and Southern analysis was conducted to confirm that the correct disruption had occurred. To construct strains TM165 and TM166, the 0.7-kb *Nde*I-*Ase*I segment of the *PTC1* gene was replaced with the 2.0-kb *Hpa*I-*Sal*I fragment of *LEU2*.

Expression and purification of the GST-PTC1 fusion protein. An overnight culture of *E. coli* NM522[pGEX2T] or NM522[pGEX2T-PTC1] was diluted 100-fold into 1 liter of L broth (two 2-liter flasks each containing 0.5 liters of medium) containing ampicillin (100 μg/ml) and shaken at 37°C. At an OD_{595} of ~0.2, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM, and shaking was continued at 25°C for 12 h (OD_{595} was ~1.1). Cells were harvested and resuspended in 20 ml of a buffer consisting of 100 mM Tris-HCl (pH 7.0), 2 mM EDTA, and 0.5% 2-mercaptoethanol (the final volume of suspension was ~26 ml). The cell suspension was mixed with 2.5 ml of lysozyme (5 mg/ml), incubated at 14°C for 10 min, and treated by three cycles of freezing in a dry ice-ethanol bath and thawing at 14°C. Next, 320 μl of 1 M $MgCl_2$ and 320 μl of DNase I (5 mg/ml) were added to the cell extract, and the mixture was incubated at 14°C for 20 min. The reaction was stopped by adding 3.2 ml of 0.2 M EDTA. The lysate was centrifuged at 70,000 × *g* for 30 min at 4°C, using a Sorvall T-865 rotor, and 31 ml of the supernatant was recovered (crude extract). A glutathione-Sepharose 4B (Pharmacia) column (1.5-ml bed volume) was equilibrated with phosphate-buffered saline (PBS; 10 mM phosphate, 150 mM NaCl [pH 7.3]) plus 1% Triton X-100, and 15.5 ml of the crude extract was applied. The column was washed with 20 ml of PBS, and the bound GST (or GST-PTC1) protein was eluted with 5 mM glutathione in 50 mM Tris-HCl (pH 8.0). The peak fraction (3.3 ml; 6.9 mg/ml for GST and 2.3 mg/ml for GST-PTC1), which contained ~70% of the total eluted protein, was aliquoted and stored at −80°C. The frozen samples were thawed at 14°C immediately before assaying. No change in the enzyme activity was observed 1 month after storage at −80°C.

Protein phosphatase assay. Bovine milk casein (hydrolyzed and partially dephosphorylated; Sigma) was labeled on serine residues, using [γ-^{32}P]ATP (6,000 Ci/mmol) and the catalytic subunit of bovine heart cAMP-dependent protein kinase as recommended by the supplier (Sigma). Myelin basic protein (Sigma) was labeled on tyrosine residues, using [γ-^{32}P]ATP (125 Ci/mmol) and $p56^{lck}$ tyrosine kinase as described previously (58). Radiolabeled proteins were precipitated and washed twice with 20% trichloroacetic acid, washed once with acetone, dried, and suspended in 0.2 M Tris-HCl (pH 8.0) at 5×10^5 cpm/μl for casein or at 6×10^4 cpm/μl for myelin basic protein. The standard phosphatase assay mix (50 μl) contained 60 mM Tris-HCl (pH 7.0), 0.1 mM EGTA, 10 mM 2-mercaptoethanol, ~10^5 cpm of ^{32}P-labeled substrate, appropriate amounts of the enzyme preparation, and either $MgCl_2$ or $MnCl_2$ at the indicated concentration. Samples were incubated at 25°C for various times, and the reaction was terminated by the addition of 0.75 ml of acidic charcoal mixture (0.9 M HCl, 90 mM sodium pyrophosphate, 2 mM NaH_2PO_4, 4% [vol/vol] Norit A). Samples were centrifuged in a microcentrifuge, and the amount of radioactivity in 0.4 ml of supernatant was measured by scintillation counting (58). All assays were performed at least in duplicate, and similar results were obtained in two or more independent experiments.

Nucleotide sequence accession number. The GenBank nucleotide accession number for the *PTC1* sequence reported in this paper is L14593.

RESULTS

Synthetic lethal screen for mutants whose growth is dependent on the expression of *PTP1* or *PTP2*. Initially, we constructed two yeast strains, TM44 and TM45, for the synthetic lethal screen. The two strains have in common the necessary mutations for the synthetic lethal screen used in this report: disrupted alleles of *ptp1* and *ptp2* in addition to *ade2* and *ade3* mutations (27). These strains, however, are different in several aspects, including mating type, nutrient requirements, and general genetic background. Isolation of synthetic lethal mutants from these two strains facilitated genetic analyses and also ensured that the isolated mutants are not specific to a particular genetic background. TM44

and TM45 were transformed with either of two screening plasmids: pSSP15 (YCp-type plasmid carrying P_{GAL1}-*PTP1*, *ADE3*, and *URA3*) to make TM44P1 and TM45P1 or pSSP25 (YCp-type plasmid carrying P_{GAL1}-*PTP2*, *ADE3*, and *URA3*) to make TM44P2 and TM45P2. These four transformed strains were used as the parental strains for mutant screening. Because *ade2 ADE3* mutant cells accumulate a red pigment, the parental strains (*ade2 ade3*/*ADE3*) form red colonies when the screening plasmids are maintained by selection for Ura^+. Without the selection, however, these strains are apt to lose the plasmids at a certain frequency per cell division, generating colorless *ade2 ade3* cells. Thus, the resulting colonies would contain, on the red background, many white sectors consisting of cells that lack the screening plasmids. In this case, the plasmids can be lost from the parental cells, because no gene on the plasmids is essential for growth. If, however, a mutation has occurred in a chromosomal gene such that growth of the mutant cells requires expression of the plasmid-borne *PTP1* or *PTP2* gene, only those cells that carry the plasmids will survive. Such mutant cells, which cannot lose the plasmids, will form nonsectoring red colonies. Because expression of the plasmid-borne *PTP1* or *PTP2* gene is induced by galactose, the survival of *PTP*-dependent mutants will be also dependent on the presence of galactose in the media. Therefore, a test for galactose-dependent growth was used as the second-stage screen following the initial colony color sectoring assay.

Parental cells were spread on YEPGal plates and irradiated with UV light to ~5 to 20% survival, and nonsectoring cells were isolated. Those cells were retested on YEPGal plates for nonsectoring and on YEPD plates for galactose-dependent growth. From 4.0×10^4 mutagenized colonies of TM44P1 and TM45P1, three nonsectoring, galactose-dependent, *PTP1*-dependent mutants were isolated. Similarly, from 3.8×10^4 mutagenized colonies of TM44P2 and TM45P2, 22 nonsectoring, galactose-dependent, *PTP2*-dependent mutants were isolated.

Complementation tests of the *PTP*-dependent mutants. The dominance or recessiveness of the mutations that conferred *PTP* dependence was then tested. Each mutant was crossed with a parental strain of the opposite mating type, and the resulting diploid cells were tested for galactose dependence and the sectoring phenotype. For each mutant, resulting diploid cells grew on YEPD as well as on YEPGal and formed sectoring colonies on YEPGal plates. Thus, the *PTP*-dependent mutations were all recessive.

We next performed complementation tests to separate the mutants into complementation groups. The *PTP2*-dependent mutants were isolated from two parental strains of different mating types, TM44P2 (**a**) and TM45P2 (α). Thus, pairs of *PTP2*-dependent mutants isolated from different parental cells were crossed, and the resulting diploid cells were tested for galactose-dependent growth. The results indicated that the *PTP2*-dependent mutants could be categorized into three complementation groups, A, B, and C. Because all of the *PTP1*-dependent mutants were isolated from the same parental strain, TM45P1, they had the same α mating type. Thus, the complementation tests between the *PTP1*-dependent mutants could not be done. However, using the cloned genes and gene disruptant strains, we later found that all three *PTP1*-dependent mutants belong to the C complementation group of the *PTP2*-dependent mutants. The results of the complementation tests are summarized in Table 2.

Cloning and sequencing of the gene for the C complementation group. The gene for the C complementation group was cloned by screening for genomic DNA clones that rescued the galactose dependence of the group C mutants. Strain P1d5215 (originally isolated from TM45P1) was transformed with a yeast genomic DNA library, and galactose-independent transformants were selected on glucose plates. Three complementing plasmids, pPD111, pPD112, and pPD113, were identified. Similarly, pPD232 was isolated by using strain P2d5314 (originally isolated from TM45P2). Restriction mapping analysis of these plasmids revealed that three of them, pPD112, pPD113, and pPD232, had indistinguishable restriction maps and perhaps are the same plasmid. The restriction map of pPD111 was different from but overlapping with that of the other three plasmids.

TABLE 2. Complementation tests of *PTP*-dependent mutants

Complementation group	No. of mutants isolated from parental strain:				Total
	TM44P1	TM45P1	TM44P2	TM45P2	
A	0	0	1	11	12
B	0	0	1	4	5
C (*ptc1*)	0	3	2	3	8

To localize the gene responsible for the complementation, various subclones were constructed from pPD111 and pPD112, and their abilities to complement the group C mutations were tested. The results indicated that the complementing activity was localized to a 1.5-kb *Hin*dIII-*Sal*I fragment (Fig. 2).

The nucleotide sequence of the 1.5-kb *Hin*dIII-*Sal*I fragment was determined on both strands. Within this fragment, a long open reading frame that would encode a protein of 281 aa was identified (Fig. 3). Significantly, the noncomplementing subclone pPD114 contains only a portion of this coding region (Fig. 2). There was no other open reading frame longer than 100 aa in this DNA fragment.

A search of the GenBank data base for similar sequences revealed that a significant homology is found between the 281-aa protein and a mammalian serine/threonine-specific protein phosphatase, PP2C. Thus, we named the yeast gene *PTC1* (phosphatase 2 C). The deduced PTC1 amino acid sequence is compared with sequences of the mammalian counterparts, PP2Cα and PP2Cβ, in Fig. 4. The amino acid sequence of the PTC1 protein is ~35% identical to the N-terminal sequence of either PP2Cα or PP2Cβ, whereas PP2Cα and PP2Cβ sequences are 76% identical. PTC1 (281 aa) is substantially shorter at the C-terminal region than the mammalian PP2Cα (382 aa) and PP2Cβ (390 aa).

To confirm that the group C mutants have mutations in the *PTC1* gene, we carried out complementation tests between the group C mutants and the *PTC1*-disrupted strain TM165 or TM166 (Table 1). None of the group C mutants complemented the *PTC1* disruptant, indicating that they contain a mutation in the *PTC1* gene.

Disruption of the *PTC1* gene shows a synthetic growth defect with *ptp2* but not with *ptp1*. To examine whether the phenotype of the original *ptc1* mutants was caused by a loss of function rather than a gain of function, disruption of the *PTC1* gene was performed. A 0.7-kb *Nde*I-*Ase*I fragment of *PTC1*, which encompasses most of the coding sequence, was replaced with the *URA3* gene (Fig. 2). This disrupted allele (*ptc1*::*URA3*) was transplaced into a diploid strain, TM102, to make the heterozygous disruptant strain TM116. The independent isolates of the TM116 strain were induced to sporulate, and tetrads were dissected. All four spores of each tetrad gave rise to colonies of approximately identical

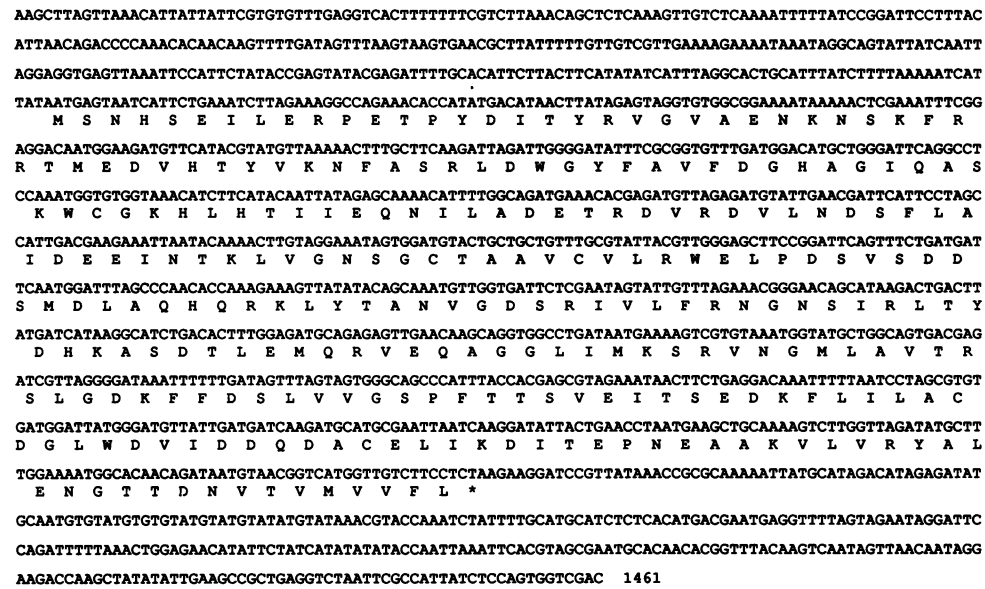

FIG. 3. Nucleotide sequence of the *S. cerevisiae PTC1* gene. The nucleotide sequence of the 1.5-kb *Hin*dIII-*Sal*I segment that complemented the *ptc1* mutations was determined on both strands. The translated amino acid sequence is shown in the standard one-letter code below the nucleotide sequence. Nucleotide positions (numbered from the *Hin*dIII site) and amino acid positions are shown at left. *, stop codon.

size on YEPD plates at 30°C (data not shown). For each tetrad, segregation of the markers (Ura, Trp, His, and mating type) was almost always 2:2. Thus, the null *ptc1* mutant cells grow normally at 30°C. Next, the null *ptc1* mutation was examined for synthetic lethality with *ptp1* and *ptp2*. For this purpose, TM126 (*ptc1*::*URA3*) was crossed with TM103 (*ptp1*::*LEU2*) or TM107 (*ptp2*::*LEU2*). After being induced to sporulate, tetrads were dissected and individual spores were examined for viability on YEPD plates at 30°C. Tetrads from the TM126 (*ptc1*::*URA3*) × TM103 (*ptp1*::*LEU2*) cross gave rise to four viable spores; four colonies from each tetrad were of approximately equal size (Fig. 5A). This result indicated that the *ptc1 ptp1* double-mutant cells grew normally. In contrast, tetrads from the TM126 (*ptc1*::*URA3*) × TM107 (*ptp2*::*LEU2*) cross gave distinct tetrad patterns (Fig. 5B). In most tetrads, one or two colonies were extremely small. By examining the Ura and Leu markers of each colony, we found that all of the small colonies had the Ura$^+$ Leu$^+$ phenotype, indicating that they had the *ptc1 ptp2* double-mutant genotype. Thus, it was concluded that the combination of *ptc1* and *ptp2* severely impaired the growth of the double mutants. In other words, the null mutations *ptc1* and *ptp2* cause a synthetic growth defect.

The results of the synthetic lethal screen indicated that overexpression of the *PTP1* gene suppresses the growth defect of the *ptc1 ptp2* double mutants (see Discussion for more details). Therefore, it is conceivable that the residual growth of the *ptc1 ptp2* double mutants is due to expression of the *PTP1* gene. To test this possibility, *ptc1/PTC1 ptp1/ptp1 ptp2/PTP2* heterozygous diploid cells were sporulated, and the dissected tetrads were analyzed. The results showed that growth of the *ptc1 ptp1 ptp2* triple mutant is even slower than growth of the *ptc1 ptp2* double mutant (data not shown).

In addition to *PTP1* and *PTP2*, another *S. cerevisiae* gene,

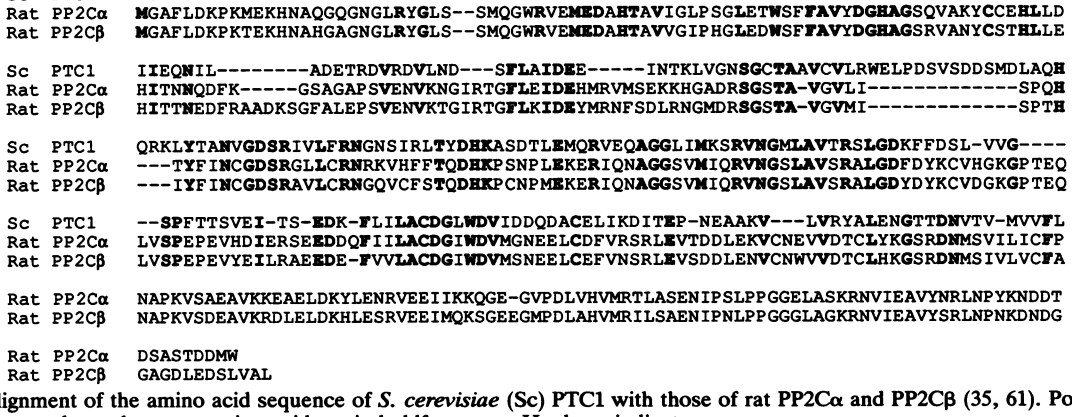

FIG. 4. Alignment of the amino acid sequence of *S. cerevisiae* (Sc) PTC1 with those of rat PP2Cα and PP2Cβ (35, 61). Positions where all three sequences have the same amino acid are in boldface type. Hyphens indicate gaps.

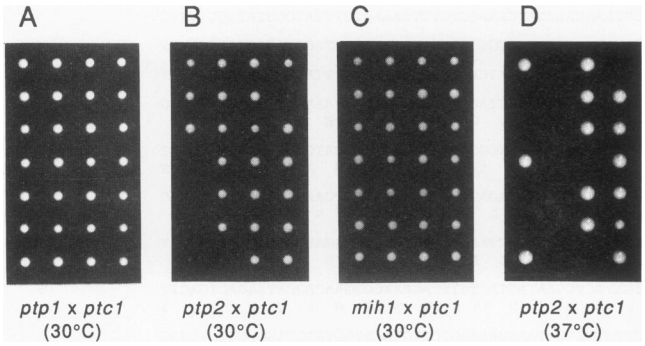

FIG. 5. Synthetic growth defect of *ptc1 ptp2* double mutants. Heterozygous diploids *ptc1*::*URA3*/*PTC1 ptp1*::*LEU2*/*PTP1* (A), *ptc1*::*URA3*/*PTC1 ptp2*::*LEU2*/*PTP2* (B and D), and *ptc1*::*URA3*/*PTC1 mih1*::*LEU2*/*MIH1* (C) were sporulated, and the dissection plates were incubated at 30°C. Examination of the Ura and Leu phenotypes indicated that the sizes of the colonies of the *ptc1 ptp1* or *ptc1 mih1* double mutants did not differ from those of the *ptc1*, *ptp1*, or *mih1* single mutants (A and C). In contrast, colonies of the *ptc1 ptp2* double mutants were much smaller than those of the *ptc1* or *ptp2* single mutants (B). The *ptc1* mutant cells failed to grow at 37°C, irrespective of the genotype of the *PTP2* gene (D).

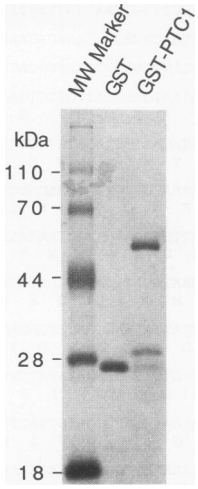

FIG. 6. Purified GST-PTC1 fusion protein. Five micrograms of purified GST-PTC1 or GST was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide–0.33% bisacrylamide). After the gel was fixed, proteins were stained with Coomassie brilliant blue. Molecular weights (MW) are indicated at the left.

MIH1, is known to encode an enzyme with tyrosine phosphatase activity (49). *MIH1* is a homolog of the *S. pombe cdc25* gene, which is believed to dephosphorylate *cdc2* kinase. The structural similarity of MIH1 to PTP1, PTP2, or mammalian PTPases is restricted to a few amino acids around the catalytic center. Unlike the *S. pombe cdc25* mutants, *S. cerevisiae mih1* mutants have no distinguishable phenotype (49). Furthermore, the *ptp1 mih1* and *ptp2 mih1* combinations are viable (19). To examine whether *mih1* and *ptc1* are synthetically lethal, TM126 (*ptc1*::*URA3*) was crossed with TM111 (*mih1*::*LEU2*), and tetrads were dissected. Because all four spores formed colonies of approximately the same size, the *ptc1 mih1* double mutants grow normally (Fig. 5C).

To test whether the incubation temperature has any effect on growth of the *ptc1* mutants, the tetrads from the *ptc1*/*PTC1 ptp2*/*PTP2* heterozygous diploids were incubated at 30 and 37°C. Interestingly, both *ptc1 PTP2*$^+$ and *ptc1 ptp2* mutants formed extremely small colonies at 37°C (Fig. 5D). Thus, *PTC1* function seems to be essential for either growth or germination at 37°C.

The *PTC1* gene is tightly linked to the *TRP1* gene on chromosome IV. During the course of the tetrad analyses involving the *ptc1* mutation, we observed a tight linkage between *PTC1* and *TRP1*. Of the total of 186 informative tetrads derived from various crosses involving the *PTC1* and *TRP1* genes, 180 parental ditypes, 6 tetratypes, and no nonparental ditypes were found, indicating that the genetic distance between *PTC1* and *TRP1* is ~1.6 centimorgans. Thus, the *PTC1* gene resides on the chromosome IV near the *TRP1* gene and the centromere.

The *PTC1* product has serine/threonine phosphatase activity. To examine whether the *PTC1* gene encodes an active protein phosphatase, a GST-PTC1 fusion gene was expressed in *E. coli*. The fusion protein was purified by glutathione-Sepharose 4B column chromatography (56). From 0.5 liter of bacterial culture, ~7.6 mg of GST-PTC1 protein or 22.9 mg of GST protein was obtained (Fig. 6).

Serine/threonine phosphatase activity of the purified GST-PTC1 protein was assayed by using a casein substrate that was phosphorylated by cAMP-dependent protein kinase and [γ-^{32}P]ATP. The results indicated that in the presence of Mg^{2+} (Fig. 7A) or Mn^{2+} (Fig. 7B), GST-PTC1, but not the control GST protein, dephosphorylated the casein substrate. This phosphatase activity was absolutely dependent on the presence of the divalent cation Mg^{2+} or Mn^{2+} (Fig. 7C and D). Interestingly, in these reactions, Mn^{2+} was a substantially more effective cofactor than Mg^{2+}. The optimal Mg^{2+} concentration (>30 mM) was much higher than the optimal Mn^{2+} concentration (5 to 10 mM). Furthermore, even though the reaction with Mg^{2+} (Fig. 7A) was done with fivefold more GST-PTC1 protein than was used for the reaction with Mn^{2+} (Fig. 7B), the initial reaction velocity with Mg^{2+} was only 25% of the reaction with Mn^{2+}. Thus, under these conditions, the phosphatase reaction is about 20 times more efficient with Mn^{2+} than with Mg^{2+}.

Finally, we examined whether GST-PTC1 has any tyrosine phosphatase activity. For this purpose, the purified GST-PTC1 or the control GST preparation was incubated with tyrosine-phosphorylated myelin basic protein, and the release of P_i was measured. Figure 8 shows that PTC1 has a very weak but significant protein tyrosine phosphatase activity in the presence of Mn^{2+}. When the reaction mixture contained Mg^{2+} instead of Mn^{2+}, the tyrosine phosphatase activity of PTC1 was only about 5% of the activity measured in the presence of Mn^{2+}. Thus, the ratios of the serine phosphatase activity to tyrosine phosphatase activity are about the same (200:1) whether they were measured in the presence of Mn^{2+} or in the presence of Mg^{2+}. This finding suggests that both activities are catalyzed by a common reaction mechanism. However, because the tyrosine phosphatase activity of PTC1 is only 0.5% of its serine phosphatase activity, it is not clear whether the tyrosine phosphatase activity has any physiological significance. It should be noted that the mammalian serine/threonine phosphatases PP2A and PP2B also have weak tyrosine phosphatase activities (6, 33).

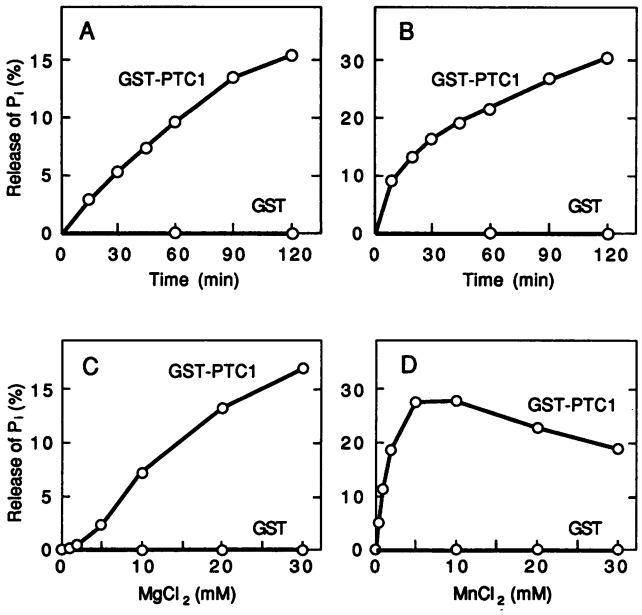

FIG. 7. Protein serine/threonine phosphatase activity of the GST-PTC1 fusion protein. Purified GST-PTC1 and GST proteins were incubated with ^{32}P-labeled casein, and the radioactivity of the released P_i was measured. Reaction conditions were as described in Materials and Methods. (A) Time course of the PTC1 reaction in the presence of 30 mM $MgCl_2$. Ten micrograms of GST-PTC1 or GST was assayed at 25°C for the indicated times. (B) Time course of the PTC1 reaction in the presence of 10 mM $MnCl_2$. Two micrograms of GST-PTC1 or 10 μg of GST was assayed at 25°C for the indicated times. (C) Effect of Mg^{2+} concentration on PTC1 activity. Ten micrograms of GST-PTC1 or GST was incubated with ^{32}P-labeled casein at 25°C for 120 min at the indicated concentrations of $MgCl_2$. (D) Effect of Mn^{2+} concentration on PTC1 activity. Ten micrograms of GST-PTC1 or GST was assayed at 25°C for 15 min at the indicated concentrations of $MnCl_2$.

DISCUSSION

In this report, we identified three genes that, when inactivated, make yeast growth dependent on the expression of the protein tyrosine phosphatase gene *PTP2*. One of the three genes, *PTC1*, encodes the first reported yeast homolog of the mammalian protein serine/threonine phosphatase PP2C.

Mutations in the *PTC1* gene were isolated from the screen for *PTP1*-dependent mutants as well as from the screen for *PTP2*-dependent mutants. However, when a disruption in the *PTC1* gene was combined with either *ptp1* or *ptp2* mutation, a synthetic growth defect was observed only with the *ptc1 ptp2* cells, not with the *ptc1 ptp1* cells. This apparent contradiction is due to the fact that overexpression of the *PTP1* gene suppresses the growth defect of the *ptp2 ptc1* double mutants. The parental strains had the relevant genotype *ptp1 ptp2* [P_{GAL1}-*PTP1*] or *ptp1 ptp2* [P_{GAL1}-*PTP2*] (genes in brackets are carried by plasmids). The *ptc1* mutants derived from these parental strains, namely, *ptc1 ptp1 ptp2* [P_{GAL1}-*PTP1*] and *ptc1 ptp1 ptp2* [P_{GAL1}-*PTP2*], behave essentially the same. These triple mutants do not grow in the absence of galactose in the media. In the presence of galactose, both mutants grow because overexpression of either *PTP1* or *PTP2* suppressed the growth defect. Thus, *ptc1* mutants behave as if they are dependent on either *PTP1* or *PTP2* expression. However, *PTP1* expression from the single chromosomal copy is not sufficient to suppress the defect of the *ptc1 ptp2* mutants. In contrast, the chromosomal *PTP2* gene is sufficient to support the growth of the *ptc1 ptp1* mutants.

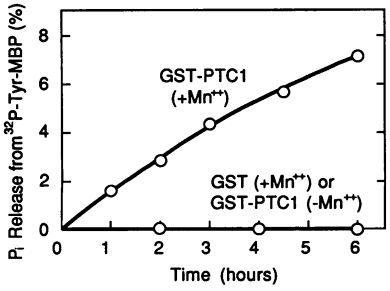

FIG. 8. Protein tyrosine phosphatase activity of the GST-PTC1 protein. Ten micrograms of GST-PTC1 or GST was assayed at 25°C for the indicated times in either the presence or absence of 10 mM $MnCl_2$, using tyrosine-phosphorylated myelin basic protein (Tyr-MBP) as the substrate.

The asymmetry in the phenotypes of *ptc1 ptp1 PTP2* and *ptc1 PTP1 ptp2* indicates functional difference between the *PTP1* and *PTP2* genes. The structural differences between PTP1 and PTP2 may account for the functional difference. PTP2 has a noncatalytic accessory domain on the N-terminal side of the catalytic domain, whereas PTP1 has no accessory domain. Furthermore, the catalytic domain of PTP2 has a unique insertion sequence, although its functional significance is unknown. Alternatively, it is possible that the expression level of the chromosomal *PTP1* gene is much lower than that of the *PTP2* gene.

An intriguing conclusion from this study is that either a tyrosine phosphatase or a serine/threonine phosphatase, but not both, is required in a signal-transducing pathway. How can be explain this apparent paradox? It might be possible that both the *PTC1* and *PTP2* phosphatases act on a common phosphotyrosine residue, because the *PTC1* enzyme has tyrosine phosphatase activity (Fig. 8). However, the physiological relevance of the limited tyrosine phosphatase activity of PTC1 might be questionable. Thus, a more realistic interpretation of our results will call for the involvement of two separate phosphates, one linked to a tyrosine residue and another linked to a serine or threonine residue. It is possible that two separate substrates exist: one for the *PTP2* tyrosine phosphatase and another for the *PTC1* serine/threonine phosphatase. Furthermore, these two substrates could serve a redundant function. Then, dephosphorylation of either substrate would fulfill the required function.

Another possible model is that the *PTP2* and *PTC1* phosphatases share a common substrate protein. Such a protein would be dually phosphorylated on a tyrosine and a serine/threonine residue, and dephosphorylation of either residue would be sufficient for cell growth. Several proteins whose activities are regulated by phosphorylation of both tyrosine and serine/threonine residues, including MAP kinases and the *cdc2* kinase, are known (2, 28, 29, 39). MAP kinases are a group of serine/threonine protein kinases that are commonly activated by various extracellular signals and are believed to be key components in kinase cascades. Activation of MAP kinases requires phosphorylation of conserved tyrosine and threonine residues. Normally, an activated MAP kinase will be inactivated by the action of phosphatases in due time. Because this inactivation can be achieved by either a tyrosine phosphatase or a serine/

threonine phosphatase, the presence of either enzyme is sufficient to regulate MAP kinase activity. However, when both tyrosine phosphatase and threonine phosphatase are absent, an activated MAP kinase will be permanently stuck in the activated state. It is likely that constitutive activation of a MAP kinase is deleterious. This scenario is reminiscent of the synthetic effect of the *PTP2* and *PTC1* phosphatases. However, we are not implying that a MAP kinase is the substrate of *PTP2*/*PTC1* phosphatases.

Mammalian protein serine/threonine phosphatases are classified into four subfamilies, PP1, PP2A, PP2B, and PP2C, based on their preferences for substrates, metal cation requirements, and sensitivities to inhibitors (8). The PP1, PP2A, and PP2B groups all belong to the same supergene family, and multiple genes have been identified for each subfamily. Furthermore, additional protein phosphatases that are distantly related to PP1, PP2A, and PP2B have been isolated from many organisms (7, 11). At least 12 serine/threonine-specific protein phosphatase genes that belong to the PP1/PP2A/PP2B supergene family have been cloned from *S. cerevisiae* (7, 45). In contrast, the PP2C enzymes comprise a separate gene family with no sequence similarity to the PP1/PP2A/PP2B phosphatases. The PP2C phosphatase is distinguished from other serine/threonine protein phosphatases by its dependence on Mg^{2+} (or Mn^{2+}) and its resistance to okadaic acid (9). In mammalian cells, two PP2C isozymes (PP2Cα and PP2Cβ) have been cloned (35, 61, 63), but their physiological function is unknown. Although no yeast homolog of PP2C was cloned previously, Cohen et al. had reported a PP2C-like phosphatase activity (i.e., okadaic-acid resistant, Mg^{2+}-dependent protein serine/threonine phosphatase) in *S. cerevisiae* cell lysates (10). Our results unambiguously prove that *S. cerevisiae* has a protein phosphatase homologous to mammalian PP2C.

Nevertheless, we do not think that the previously reported PP2C-like activity corresponds to the *PTC1* enzyme, for the following reasons. The Mg^{2+} concentration required for half-maximal activation is nearly 10-fold higher for PTC1 (~10 mM) than for the PP2C-like activity in yeast cell extracts (10). Furthermore, the PP2C-like phosphatase activity in the cell extracts of the *ptc1* mutants was not different from that of the parental strain (34). These observations thus suggested the possibility that there are additional PP2C homologs in *S. cerevisiae*. Indeed, using the polymerase chain reaction and cross-hybridization, we have cloned two additional PP2C homologs, PTC2 and PTC3, from *S. cerevisiae* (34). Because neither *PTC2* nor *PTC3* can complement the *ptc1* defect, *PTC1* seems functionally distinct from *PTC2* and *PTC3*. By analogy, it might be expected that mammalian cells also have more members of the PP2C family than the two known isozymes. Identification of members of the PP2C family in *S. cerevisiae* provides a new way to study the physiological function of the PP2C phosphatases.

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