Counteractive roles of protein phosphatase 2C (PP2C) and a MAP kinase kinase homolog in the osmoregulation of fission yeast

Kazuhiro Shiozaki and Paul Russell¹

Departments of Molecular and Cell Biology, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA

¹Corresponding author

Communicated by P.Nurse

With the goal of discovering the cellular functions of type 2C protein phosphatases, we have cloned and analyzed two *ptc* (phosphatase two C) genes, *ptc2*⁺ and *ptc3*⁺, from the fission yeast *Schizosaccharomyces pombe*. Together with the previously identified *ptc1*⁺ gene, the enzymes encoded by these genes account for ~90% of the measurable PP2C activity in fission yeast cells. No obvious growth defects result from individual disruptions of *ptc* genes, but a $\Delta ptc1 \Delta ptc3$ double mutant displays aberrant cell morphology and temperature-sensitive cell lysis that is further accentuated in a $\Delta ptc1 \Delta ptc2 \Delta ptc3$ triple mutant. These phenotypes are almost completely suppressed by the presence of osmotic stabilizers, strongly indicating that PP2C has an important role in osmoregulation. Genetic suppression of $\Delta ptc1 \Delta ptc3$ lethality identified two loci, mutations of which render cells hypersensitive to high-osmolarity media. One locus is identical to *wis1*⁺, encoding a MAP kinase kinase (MEK) homolog. The Wis1 sequence is most closely related to the *Saccharomyces cerevisiae* MEK encoded by *PBS2*, which is required for osmoregulation. These data indicate that divergent yeasts have functionally conserved MAP kinase pathways, which are required to increase intracellular osmotic concentrations in response to osmotic stress. Moreover, our observations implicate PP2C enzymes as also having an important role in signal transduction processes involved in osmoregulation, probably acting to negatively regulate the osmosensing signal that is transmitted through Wis1 MAP kinase kinase

Key words: fission yeast/MAP kinase cascade/osmoregulation/protein phosphatase 2C

Introduction

Cells operate effectively under a relatively narrow range of environmental conditions. Exposure to extremes of temperature, pH, osmolarity and many other conditions induce dramatic cellular changes that serve to ensure the maximum probability of recovery and survival. The changes that occur in response to stress are pleiotropic, encompassing transcription, translation, protein modifications and degradation and other aspects of cellular metabolism. The stress response is also extremely rapid and therefore reversible protein phosphorylation is viewed as

a prime candidate mechanism for signal transduction in the stress response.

Protein serine/threonine phosphatases are classified into four major classes: types 1 (PP1), 2A (PP2A), 2B (PP2B) and 2C (PP2C), by their substrate specificity, divalent cation requirements and sensitivity to inhibitors (Cohen, 1989). PP2C is unique in requiring magnesium and being insensitive to certain phosphatase inhibitors, such as okadaic acid. Genes encoding two isozymes of PP2C have been identified in mammals (Tamura *et al.*, 1989; Mann *et al.*, 1992; Wenk *et al.*, 1992) and, subsequently, several homologous genes have been isolated in various organisms, such as the budding yeast *Saccharomyces cerevisiae* (Maeda *et al.*, 1993, 1994; Robinson *et al.*, 1994), fission yeast (Shiozaki *et al.*, 1994), *Leishmania* (Burns *et al.*, 1993) and *Arabidopsis* (Leung *et al.*, 1994; Meyer *et al.*, 1994). Pyruvate dehydrogenase phosphatase (PDP) in bovine mitochondria also shows significant similarity to this class of enzymes (Lawson *et al.*, 1993). These PP2C enzymes have no apparent sequence homology to the other classes of phosphatases, while types 1, 2A and 2B enzymes share ~40% identity in their catalytic domains (Cohen and Cohen, 1989). The uniqueness of PP2C is also seen in its subunit structure; this class of enzymes exist as monomeric proteins, with the exception of PDP, which is a heterodimer of the 50 and 97 kDa subunits (Pratt *et al.*, 1982; Teague *et al.*, 1982). Enzymes of the other three classes of serine/threonine protein phosphatases form oligomeric structures with regulatory subunits which are thought to be responsible for regulation of activity, substrate specificity or cellular localization (Cohen, 1989; Cohen and Cohen, 1989).

While major advances have recently been made in discovering the biological functions of most types of protein phosphatases (reviewed by Mumby and Walter, 1993), a lack of genetic approaches and the absence of specific inhibitors have greatly hindered the analysis of PP2C enzymes. However, recent studies have suggested intriguing connections between PP2C enzymes and several cellular signal transduction systems. The *Arabidopsis ABI1* gene, which is required for the proper cellular response to the hormone abscisic acid, was recently found to encode an unusual PP2C-like protein with a putative Ca²⁺ binding site (Leung *et al.*, 1994; Meyer *et al.*, 1994). Two PP2C genes in *Saccharomyces cerevisiae*, *PTC1* and *PTC3*, were isolated as high copy suppressors of *sln1* mutations (Maeda *et al.*, 1994). The *SLN1* gene encodes a transmembrane protein homologous to the histidine kinases and the response regulator proteins of bacterial two-component systems, which have been identified in a wide variety of signaling pathways (Ota and Varshavsky, 1993).

We identified a PP2C gene in the fission yeast *Schizosaccharomyces pombe*, *ptc1*⁺, as a multicopy suppressor of the temperature-sensitive (*ts*) *swol-26* mutation (Shiozaki

TTTGATTCTCGAGATCTGGGTTTCGACTCCAGTACGACCTTTTGGCCAGAGCGAACAGTGAAGGAAAGAAGCAAGAATTGCTTTTAGACCTGGGTTTG	-496
GATTACTTTTTTCGATATCGCCATAGCTTTATATATAAGTAAGGGCTCCCTCGATGCGCTAGCAATTTCAAGGCATCGTTTATTAGTGTCTTTGTGAGGGT	-397
ATATTAGCCGGAGCTGGAGCACATTGAAGCTTAAACGATCAAAATTTATTTTATATTGTTAAATCATTCTACTAAATATTATCCCAACCTTCATT	-298
TTACGCGCTTTTCTACCCAACAACGATTGGTTAATAACGTAAGCGGTACGAATTTGCAAAGCAACGCTTCCGTGGTGTACAGAAAATCGCTATTTTTTA	-199
ACGAGACAACCTTTAAAGCGTAATGCGATATAAAGTTTTTTTAAAGTATCCTTATATATATTATTAGCTAGCGTGGTTAAATTCCCTATTAATTTGCTCAT	-100
ACACTTTTTTTTGGTTCCCTTTCCGTAACTTTACTAAACGCATCAACCGCAAATACAGGTTTTTTTAGCTGGGCTAACAAAGGTAATAGAAAATATACAC	-1
ATG GGC CAA ACA CTC TCA GAA CCC GTA CTC GAC AAA CAT S AGT TCT GGC GGG GAT CGA TGG CTT CAT TTT GGC	75
M G Q T L S E P V L D A A H T S S G G D R W L H F G	25
GTT TCA CAC ATG CAA GGA TGG CGT ATT TCG ATG GAA GAC GCG CAT TGT GCG TTG CTA AAC TTT ACG GAT TCC AAT	150
V S H M Q G W R I S M E D A H C A L L N F T D S N	50
TCT TCT AAT CCT CCG ACA AGC TTC TTT GGG GTC TTT GAT GGG CAC GGA GGC GAC AGA GTC GCC AAG TAC TGC AGG	225
S S N P P T S F F <u>G V F D G H</u> G G D R V A K Y C R	75
CAG CAC TTA CCA GAT ATC ATT AAA AGT CAG CCG TCT TTT TGG AAG GGT AAC TAT GAT GAA GCT TTG AAA TCT GGT	300
Q H L P D I I K S Q P S F W K G N Y D E A L K S G	100
TTT TTG GCT GCA GAC AAT GCG TTA ATG CAA GAT CGG GAC ATG CAG GAA GAT CCT TCG GGA TGC ACA GCT ACC ACT	375
F L A A D N A L M Q D R D M Q E D P S G C T A T T	125
GCC TTG ATT GTC GAC CAT CAA GTA ATA TAT TGT GCA AAT GCT GGG GAC TCG CGA ACG GTC TTA GGT AGA AAG GGA	450
A L I V D H Q V I Y C A N A G D S R T V L G R K G	150
ACT GCG GAA CCA CTT TCT TTT GAT CAC AAA CCA AAC AAC GAC GTG GAA AAA GCT CGA ATC ACG GCT GCA GGG GGA	525
T A E P L S F D H K P N N D V E K A R I T A A G G	175
TTT ATC GAC TTT GGT CGA GTG AAT GGT AGT TTG GCT CTT TCT CGT GCC ATT GGA GAC TTT GAG TAC AAG AAA GAC	600
F I D F G R V N G S L A L S R A I G D F E Y K K A D	200
AGT TCC TTA CCT CCA GAA AAA CAG ATC ATC GTT ACA GCT TTT CCT GAT GTC GTT ATT CAT AAC ATA GAT CCA GAT GAT	675
S S L P P E K Q I V T A F P D V V I H N I D P D D	225
GAA TTT TTA ATT CTG GCC TGT GAT GGA ATT TGG GAT TGC AAG AGC TCT CAA CAA GTC GTG GAG TTT GTA AGA AGA	750
E F L I L A <u>C D G I W D C</u> K S S Q Q V V E F V R R	250
GGC ATT GTT GCG CGG CAG TCT TTG GAA GTA ATT TGT GAA AAT CTT ATG GAC CGT TGT ATT GCT AGT AAC AGC GAG	825
G I V A R Q S L E V I C E N L M D R C I A S N S E	275
TCC TGC GGT ATC GGA TGT GAC AAC ATG ACT ATT TGC ATT GTT GCC TTT TTG CAT GGT CGT GGT CTT GAG GAT TGG	900
S C G I G C D N M T I C I V A F L H G R G L E D W	300
TAT AAT TGG ATT ACA CAG CGC GTC AAC TCG GGA GAA GGT CCT TGC GCA CCT CCA TCT TAT GCT GAG TTG CGA GGA	975
Y N W I T Q R V N S G E G P C A P P S Y A E L R G	325
CCT AAC ACT ATA GCG GAT GCA AGA AAT CTG CAG TTG GAA TAT GAT CAT ATA GCT AGC CAT GAG TAT GGA AGC GGT	1050
P N T I A D A R N L Q L E Y D H I A S H E Y G S G	350
GAT ACA TAC GAT AGC GAT TCA GAT GAT GAA ACC ATT GCC TAC GAT CGC TAT TAC CTT CAT TAA GTTATTTTCATTGCT	1128
D T Y Y D S D S D D E T I A Y D R T Y L H *	370
TTTTTTTTTAAAGCAATGAAGGGTTTGTGTTGTTGTTGTTTTCGTATAACATCCTATCTCAGCAAGTTATAACAGGATTCTCTTCTTTTTTAAATT	1227
TTCAATTACTTTCTTTCCCTTCCTTCCTTTTCTCAAGTTTTTCAGGCTTCTCGCATCGTTTTTAATAAATAGTTTCCCTCTTTATTCGGGAAAAATA	1326

Fig. 1. The nucleotide sequence of *ptc2*⁺ and predicted amino acid sequence. The regions corresponding to the PCR primers are underlined. The EMBL Data Library accession number of this sequence is L34881.

et al., 1994). *swol*⁺ encodes a heat shock protein closely related to hsp90 in higher eukaryotes and *swol* mutant cells are swollen and lysed at restrictive temperature. *ptc1*⁺ expression is induced by heat shock and *ptc* deletion mutant cells die more quickly than do wild-type cells after shifting to 48°C, suggesting a role for *ptc1*⁺ in the heat shock response. Measurement of PP2C activity in cell lysates of the *ptc1* deletion mutant demonstrated that *ptc1*⁺ contributes only a minor portion of the cellular PP2C activity, indicating that fission yeast has multiple PP2C genes. Here, we report the isolation and characterization of two additional *S.pombe* PP2C genes, *ptc2*⁺ and *ptc3*⁺. These three PP2C genes account for most of the PP2C activity in fission yeast cells. They have overlapping functions which are important for maintaining cell shape and osmotic stability. Moreover, mutations in a MAP kinase kinase homolog, *wis1*⁺, were found to suppress the Δ *ptc1* Δ *ptc3* phenotypes. Our data strongly indicate that *ptc1*⁺, *ptc2*⁺ and *ptc3*⁺ play important roles in the osmosensing signal transduction pathway.

Results

Isolation of new PP2C homologs in *S.pombe*

Two conserved regions of the protein products of *S.pombe* *ptc1*⁺ (Shiozaki *et al.*, 1994) and mammalian PP2C genes (Tamura *et al.*, 1989; Mann *et al.*, 1992; Wenk *et al.*, 1992) were chosen to design degenerate oligonucleotide primers for PCR. The sequence AV(F/Y)DGH (residues 106–111 in *ptc1*⁺ and 57–62 in rat PP2C α) was chosen for the 5' primer, while CDG(IL)WDV (274–280 in *ptc1*⁺ and 238–243 in rat PP2C α) was chosen for the 3' primer (see Materials and methods). DNA fragments of 0.5 and 0.8 kb were specifically amplified from *S.pombe* genomic DNA prepared from a Δ *ptc1* strain (Shiozaki *et al.*, 1994). DNA sequence and Southern hybridization analyses revealed that these fragments were derived from loci encoding PP2C homologs in *S.pombe* (data not shown). The 0.5 kb PCR fragment was used to isolate a plasmid harboring *ptc2*⁺ from an *S.pombe* genomic library. The *ptc2*⁺ open reading frame encodes a 370 amino acid

TGTTACCCCTCCATAGTCTTTTAACTATCGTTCGTCTCCACGGTGGTGATGTAGTGTAGAGGGTGCCACAACACTCCCTCCGACAAGCTCCACC	-100
ACCTACTAGCGATTACCTATCACTACTACATAACCCGTTTGAAGCGACACAAAAAGAAACGTGAAAATTTGTGTCTATCTATAGCAAGAGCTGTCA	-1
ATG GGT CAG ACA TTA TCA GAG CCA GTC ACC GAG AAG CAC TCG GTG AAC GGG AGC AAT GAG TTC GTC CTT TAT GGA	75
M G Q T L S E P V T E K H S V N G S N E F V L Y G	25
CTT TCA AGC ATG CAA GGA TGG CGT ATC TCA ATG GAA GAT GCT CAT TCT GCT ATA CTT TCA ATG GAG TGC TCT GCA	150
L S S M Q G W R I S M E D A H S A I L S M E C S A	50
GTA AAA GAC CCG GTC GAC TTT TTT GCG GTT TAT GAC GGC CAC GGT GGA GAC AAA GTC GCG AAA TGG TGT GGA AGC	225
V K D P V D F F <u>A V Y D G H</u> G G D K V A K W C G S	75
AAT CTT CCT CAG ATT CTT GAA AAG AAT CCT GAT TTT CAG AAA GGC GAT TTT GTT AAT GCT TTG AAA TCT TCA TTT	300
N L P Q I L E K N P D F Q K G D F V N A L K S S F	100
TTA AAT GCT GAT AAA GCT ATT TTG GAT <u>Ggtaagttt</u> gtgtgtaacaatgataaagttgttttcattgatatacataaccattttaaagtcg	390
L N A D K A I L D D	110
ccaaaaaattgatgaattttgtgttgaaatttttatagattttttgttctttgtctagtttaatgcttagaaaaattgttaccagataatagaaatg	489
intron 1 (258 bp)	
tttacctcttgatgttgaaaagttgttacatttagtttggtttcagaaaaaaattgttcatttcactacattgcattttgctaaactattttgacagAT	588
GAT CAA TTT CAT ACG GAT CCA TCA GGT TGC ACG GCC ACT GTT GTT CTT CGT GTT GGA AAT AAA CTT TAT TGTgtaa	664
D Q F H T D P S G C T A T V V L R V G N K L Y C	134
gtcttgggaagttgttttagtcttactaattgtgttagGCT AAT GCC GGT GAC TCA AGA ACT GTC CTT GGC AGT AAG GGA ATT GCA	747
intron 2 (39 bp)	
AAA CCT CTA TCA GCA GAC CAT AAG CCT TCA AAT GAA GCC GAA AAA GCC CGT ATT TGT GCG GCT GGT GGC TTT GTA	822
K P L S A D H K P S N E A E K A R I C A A G G F V	175
GAC TTT GGC CGT GTA AAT GGA AAT TTG GCT TTG TCA AGA GCT ATC GGT GAT TTT GAG TTC ACA AAC AGT AAT TTA	897
D F G R V N G N L A L S R A I G D F E F T N S N L	200
GAA CCA GAA AAA CAA ATA GTT ACT GCC TTA CCG GAT GTT GTT GTA CAT GAG ATT ACT GAC GAT GAT GAA TTC GTC	972
E P E K Q I V T A L P D V V V H E I T D D D E F V	225
GTA CTC GCT TGT GAT GGT ATT TGG GAT TGC AAG ACT TCG CAG CAA GTG ATC GAG TTT GTT AGA CGT GGC ATA GTT	1047
V L A <u>C D G I W D C</u> K T S Q Q V I E F V R R G I V	250
GCT GGA ACC TCT CTT GAG AAG ATT GCT GAA AAT TTA ATG GAT AAC TGC ATA GCC AGT GAC ACC GAA ACT ACC GGC	1122
A G T S L E K I A E N L M D N C I A S D T E T T G	275
CTT GGT TGT GAC AAC ATG ACT GTT TGT ATA GTT GCC TTA TTG CAA GAA AAC GAT AAA TCA GCA TGG TAT AAG AAG	1197
L G C D N M T V C I V A L L Q E N D K S A W Y K K	300
ATT GCA GAT AGA GTT GCC GCT AAC GAC GGA CCC TGC GCT CCT CCT GAG TAT GCC GAA AAT CAT GGC CCA GGA TGG	1272
I A D R V A A N D G P C A P P E Y A E N H G P G W	325
CGT AGT GGC GAT AAC AAT AAG AAA GTT ATT GTC CCA CCT AAT TTT CAT CAA GTA AAG TTA AAT GGA TCT GAT GGA	1347
R S G D N N K K V I V P P N F H Q V K L N G S D G	350
TAT GAC AAA GAT GCG AAT GAA AAT TCT AAG GAA GAT GAT TCC ACC AAC GGA TCC CTT GCA GCT GGG TTC AGA TGG	1422
Y D K C D A N E N S K E D S T N G S L A A G F R W	375
AAA GAA CAC TTT TTC CCT CAT AAA GCC GAG GAG GAA AAT TCT TCA AGT GAA ACC GAT ATC GTA AAT TCT AAC AAA	1497
K E H F F P H K A E E E N S S S E T D I V N S N K	400
GAT GTT GCG GAT GAT CAT AAG GAG GCT GTG TCT GCA GCA GAT TGA TTATTCCTACTTCACGCATATTACACAATGTAACCTGTC	1581
D V A D D H K E A V S A A D *	414
TTTTAGGAATATTCCTCGTAATTAGCATTATTTGTTGACTTATTATAAAAGTCACGCTAGGCGGTTTGTGTTCAACATTTTTCATGCTTTTCTTTTGT	1680

Fig. 2. The nucleotide sequence of *ptc3*⁺ and predicted amino acid sequence. The ORF is interrupted by two predicted introns (shown by lower case) which contain *S.pombe* intron consensus sequences (bold face; Prabhala *et al.*, 1992). The regions corresponding to the PCR primers are underlined. The EMBL Data Library accession number of this sequence is L34882.

protein with a predicted M_r of 42.5 kDa (Figure 1). The 0.8 kb PCR fragment hybridized to genomic clones which contained another PP2C homolog, *ptc3*⁺ (Figure 2). The *ptc3*⁺ gene has a 1539 bp open reading frame, encoding a protein with 414 amino acids (47.6 kDa), which is interrupted by two predicted introns of 258 bp and 39 bp. These introns are surrounded by short sequence motifs (shown by bold type in Figure 2) characteristic of *S.pombe* introns (Prabhala *et al.*, 1992).

The predicted amino acid sequences of Ptc2 and Ptc3 show significant homology with other PP2C proteins (Figure 3 and Table I). The central regions of Ptc2

(residues 32–292) and Ptc3 (32–289) are 66% identical to each other and 37% identical to the corresponding region (residues 32–292) of rat PP2C α (Tamura *et al.*, 1989). Interestingly, the N-terminal 31 residues of Ptc2, Ptc3 and rat PP2C also share ~50% identity (Figure 3), while this region is not conserved in *S.pombe* Ptc1 (Shiozaki *et al.*, 1994). Moreover, the central region of *S.pombe* Ptc1 (residues 81–323) is more similar to budding yeast *PTC1/TPD1* gene products (45% identical) than it is to *S.pombe* Ptc2 and Ptc3 (~32% identical) (Table I). These observations indicate specialization of PP2C subtypes that is conserved between highly divergent organisms. The

Fig. 3. (A) Comparison of the predicted amino acid sequences of *ptc2*⁺, *ptc3*⁺ and rat PP2Cα (Tamura *et al.*, 1989). Identical amino acid residues are shaded, and residues conserved in the three sequences are boxed. **(B)** A schematic representation of the primary structures of the *S.pombe* PP2C homologs, *S.cerevisiae* PTC1/TPD1 (Maeda *et al.*, 1993; Robinson *et al.*, 1994) and rat PP2C. These PP2C genes share homologous central regions, which are shown as shaded boxes (identity is shown by %; see also Table I). The N-terminal 31 residues of *ptc2*⁺, *ptc3*⁺ and rat PP2C (hatched boxes) are highly conserved (~40% identity). The black boxes denote the regions 47% identical between *ptc2*⁺ and *ptc3*⁺. The solid lines denote non-homologous regions.

similarity between Ptc2 and Ptc3 extends downstream of the central regions (filled boxes in Figure 3B), which are followed by relatively hydrophilic C-terminal regions.

Gene disruption of *ptc2*⁺ and *ptc3*⁺

To investigate the *in vivo* functions of *ptc2*⁺ and *ptc3*⁺ gene products, one-step gene disruptions (Rothstein, 1983) of these genes were performed. Using a diploid strain of *S.pombe*, the *ptc2*⁺ or *ptc3*⁺ loci were substituted with gene fragments disrupted with the *S.pombe* *ura4*⁺ marker (Figure 4). The heterozygous diploids were sporulated and the tetrads were dissected. The haploid strains carrying either a *ptc2* deletion (Δ *ptc2*) or a *ptc3* deletion (Δ *ptc3*) were found to be viable. Four spores were viable in most of the tetrads and the segregation of the Ura marker was 2⁺:2⁻ (data not shown). By Southern hybridization it was confirmed that the Ura⁺ segregants had only disrupted copies of the genes (Figure 4).

Table I. Sequence identity between the central regions of PP2C (%)

	<i>ptc2</i> ⁺	<i>ptc3</i> ⁺	<i>PTC1/TPD1</i>	Rat PP2Cα
<i>ptc1</i> ⁺	33	31	45	28
<i>ptc2</i> ⁺		66	25	37
<i>ptc3</i> ⁺			34	37
<i>PTC1/TPD1</i>				32

The amino acid sequence identity of the central regions of *S.pombe* *ptc1*⁺ (81–323), *ptc2*⁺ (32–292) and *ptc3*⁺ (32–289), *S.cerevisiae* PTC1/TPD1 (30–282) and rat PP2Cα (32–292) are shown by percentage.

A cross between Δ *ptc2* and Δ *ptc3* was carried out which revealed that the Δ *ptc2* Δ *ptc3* double mutant is also viable. The cross produced four viable spores and haploid segregants lacking both of the genes were isolated. We further performed crosses to construct the double and triple deletion mutants containing Δ *ptc1* (Shiozaki *et al.*, 1994). All of the combinations of the mutation tested (Δ *ptc1* Δ *ptc2*, Δ *ptc1* Δ *ptc3* and Δ *ptc1* Δ *ptc2* Δ *ptc3*) were viable under normal growth conditions (see below) and they did not show any obvious defects in conjugation, meiosis or sporulation (data not shown).

Ptc1, Ptc2 and Ptc3 account for ~90% of PP2C activity in *S.pombe* cell extracts

PP2C activity in *S.pombe* cell extracts can be measured as Mg²⁺-dependent casein phosphatase activity with okadaic acid added to inhibit PP2A (Kinoshita *et al.*, 1990; Shiozaki *et al.*, 1994). By MonoQ chromatography, the activity in the wild-type cell lysate separated into two broad peaks (peak fractions in samples 5 and 11 in Figure 5A). The second peak has a prominent shoulder (fraction ~16 in Figure 5A). To identify the activities of Ptc2 and Ptc3, cell lysates were prepared from Δ *ptc2* and Δ *ptc3* strains and analyzed in the same way. Whereas no significant change was obvious in the Δ *ptc2* extract, PP2C activity in the Δ *ptc3* extract showed an ~60% decrease (Figure 3A). The deletion of *ptc3*⁺ did not affect the activity in the first peak, but the second peak largely disappeared and a third peak that appeared as a shoulder in the wild-type was now evident.

Since a relatively large portion of the PP2C activity was depleted in the Δ *ptc3* disruptant, it was expected that the PP2C activities contributed by Ptc1 and Ptc2 would be more apparent in a Δ *ptc3* background. Therefore, the activity due to Ptc1 and Ptc2 was assessed by assaying the activity in extracts made from Δ *ptc1* Δ *ptc3* and Δ *ptc2* Δ *ptc3* strains. In extracts from Δ *ptc1* Δ *ptc3* cells, the first peak (fraction 5) was decreased by ~50% (Figure 5B), consistent with the earlier observations (Shiozaki *et al.*, 1994). On the other hand, the third peak (fraction 16) disappeared in the Δ *ptc2* Δ *ptc3* extract (Figure 5C); it therefore seemed to be derived from Ptc2.

Figure 6A shows PP2C activity in wild-type and Δ *ptc1* Δ *ptc2* Δ *ptc3* triple mutant cell lysates. In the triple mutant, a significant amount of activity remained in the position of the first peak, as expected from the results of Figure 5. To confirm that the activity remaining in the triple mutant is Mg²⁺-dependent, casein phosphatase activity of fractions 4–7 in the first peak (Figure 6A) was assayed in the presence of EDTA, instead of MgCl₂. The level of

Fig. 4. Gene disruption of *ptc2*⁺ and *ptc3*⁺. The *Bgl*III–*Bam*HI fragment containing the *ptc2::ura4*⁺ disrupted gene (A) and the *Hind*III fragment containing *ptc3::ura4*⁺ (B) were used to transform a diploid *S.pombe* strain. Stable Ura⁺ transformants produced four viable spores and the segregation of Ura marker was 2⁺:2[−]. The genomic DNA of the Ura[−] (lanes −) and Ura⁺ (lanes +) haploid segregants were prepared and digested by *Eco*RI (A) or *Hind*III (B). The *ptc2*⁺ *Bgl*III–*Bam*HI 2.9 kb fragment (A) and the *ptc3*⁺ *Eco*RI–*Hind*III 1.7 kb fragment (B) were used as probes for Southern hybridization to confirm gene disruptions.

Mg²⁺-independent, okadaic acid-resistant activity (Figure 6B, open columns) was very low and more than 80% of the total activity in these four fractions showed Mg²⁺ dependency (shaded columns). These results indicate that the remaining activity in the triple mutant has the characteristics of PP2C, thus there must be at least one additional PP2C gene in fission yeast. It is noteworthy that the activity in the first peak is increased in the triple mutant relative to *Δptc1 Δptc3* cells (Figure 5B), suggesting that the activity derived from the additional gene(s) may be increased to compensate for the simultaneous loss of Ptc1, Ptc2 and Ptc3 proteins.

The *Δptc1 Δptc3* double mutant shows defects in cell morphology and growth at high temperature

As the *Δptc1 Δptc2 Δptc3* triple mutant was viable at 30°C, the phenotypes of various *Δptc* mutant combinations were examined under a range of conditions. We first examined the heat shock response. The *Δptc1* deletion strain has a greatly reduced ability to survive exposure to elevated temperature; the mutant cells die more quickly after shifting from 25 to 48°C than do wild-type cells (Shiozaki *et al.*, 1994). The same experiments were performed using the *Δptc2* and *Δptc3* strains. Unlike *Δptc1*, the sensitivity to a 48°C treatment in both of the

Fig. 5. PP2C activity in the crude cell lysates of (A) wild-type (PR109) and *Δptc3* (KS948); (B) *Δptc3* and *Δptc1 Δptc3* (KS920); (C) *Δptc3* and *Δptc2 Δptc3* (KS890) strains. Wild-type and *ptc* mutant cells were grown to mid-log phase in YES medium at 30°C. The cell extracts were chromatographed on an ion-exchange column MonoQ HR5/5 and fractions were assayed for Mg²⁺-dependent casein phosphatase activity in the presence of 100 nM okadaic acid (Shiozaki *et al.*, 1994). The activity is shown by the amount of released ³²P as a percentage of [³²P]casein added in the reaction mixture.

other deletion mutants showed no significant difference from that in the wild-type strain (data not shown). The *ptc1*⁺ gene was first isolated as a multicopy suppressor of *swol-26*, a *ts* mutation of an hsp90 homolog in *S.pombe* (Shiozaki *et al.*, 1994). The *ptc2*⁺ and *ptc3*⁺ genomic clones on the multicopy vector pFL20 (Heyer *et al.*, 1986) were used to transform the *swol-26* strain. While multicopy *ptc2*⁺ did not rescue the growth defect of *swol-26*, the *swol-26* cells carrying the *ptc3*⁺ plasmid could grow well at a semi-permissive temperature of 32°C, but not at 35.5°C (data not shown).

We further examined the effect of *ptc* gene disruptions by microscopic observation under different growth conditions. The morphology of *Δptc1* and *Δptc2* cells was indistinguishable from that of wild-type cells (data not shown). On the other hand, deletion of *ptc3*⁺ caused a

Fig. 6. PP2C activity in the $\Delta ptc1 \Delta ptc2 \Delta ptc3$ triple mutant (KS917) cell lysate. (A) Mg^{2+} -dependent casein phosphatase activity in wild-type (closed squares) and the triple mutant (open squares) cell extracts were measured as in Figure 5. (B) The triple mutant fractions 4–7 in (A) were assayed again for casein phosphatase activity in reaction buffer containing either 20 mM $MgCl_2$ or 1 mM EDTA. Mg^{2+} -dependent activity (shaded columns) was calculated by subtracting the activity in the presence of EDTA (open columns) from the activity in the presence of $MgCl_2$. Okadaic acid (100 nM) was added to inhibit PP2A activity in the extracts.

decrease in cell length; the length of septated cells of $\Delta ptc3$ was 12.1 ± 0.3 (mean $\pm$ SD) μm at 30°C in YES medium versus 14.3 ± 0.5 μm in wild-type cells. Introduction of the $\Delta ptc1$ or $\Delta ptc2$ mutations into the $\Delta ptc3$ mutant did not accentuate this phenotype. However, in the cultures of the $\Delta ptc1 \Delta ptc3$ and $\Delta ptc1 \Delta ptc2 \Delta ptc3$ strains grown at intermediate temperatures (25–30°C), swollen and deformed cells were found at a high frequency (Figure 7, upper panels) and dead cells were frequently seen. Addition of 1.2 M sorbitol to the growth medium largely rescued this morphological defect and reduced the rate of cell death (Figure 7, lower panels). The cell length of the wild-type strain was shorter in this medium (septated cells 11.8 ± 0.4 μm), which was similar to that of the $\Delta ptc1 \Delta ptc3$ and $\Delta ptc1 \Delta ptc2 \Delta ptc3$ cells in the same medium (11.4 ± 0.6 μm and 11.4 ± 0.4 μm respectively).

In addition to the morphological defects, the $\Delta ptc1 \Delta ptc3$ and $\Delta ptc1 \Delta ptc2 \Delta ptc3$ cells were highly impaired

for growth at higher temperatures. At 36°C, which is within the optimum growth temperature range of *S.pombe*, $\Delta ptc1 \Delta ptc3$ cells grew very poorly, while no growth was seen with the $\Delta ptc1 \Delta ptc2 \Delta ptc3$ triple mutant (Figure 8B). Most of the cells lysed after overnight incubation at this temperature and the small number of cells that remained were highly swollen (data not shown). Addition of sorbitol to the growth medium also suppressed the *ts* growth phenotype of the $\Delta ptc1 \Delta ptc3$ and $\Delta ptc1 \Delta ptc2 \Delta ptc3$ cells. On medium containing 1.2 M sorbitol, these mutant cells formed colonies (Figure 8C) and cell shape was found to be normal (data not shown). Other osmotic stabilizers, such as 1 M galactose, 0.5 M maltose (disaccharide) or 0.5 M KCl, could also be used to suppress the *ts* phenotype of the $\Delta ptc1 \Delta ptc3$ and the triple mutant strains.

These results indicate that Ptc1 and Ptc3 activities are important for osmotic stability and cell shape control. Phenotypes arising from loss of Ptc1 and Ptc3 are accentuated at high temperatures. The functions of these genes can be compensated for by addition of osmotic stabilizers to the growth media. Since the $\Delta ptc1 \Delta ptc2 \Delta ptc3$ mutant has a more severe phenotype than the $\Delta ptc1 \Delta ptc3$ double mutant, and the *ptc2*⁺ gene on a multicopy plasmid can suppress the defects of the $\Delta ptc1 \Delta ptc3$ double mutant (data not shown), Ptc2 also appears to impact osmotic stability and cell shape control.

***Δptc* mutants have no apparent defect in cell wall construction**

We felt that the phenotypes exhibited by the $\Delta ptc1 \Delta ptc3$ double mutant were most likely due to defects either in cell wall organization or in osmoregulation. The former possibility was suggested by studies carried out with the *Saccharomyces cerevisiae* $\Delta pkc1$ mutant, which lacks a protein kinase C homolog (Levin *et al.*, 1990). The $\Delta pkc1$ mutant, which exhibited a cell lysis phenotype that was suppressed by osmotic stabilizers, had a much thinner cell wall than wild-type cells (Levin and Bartlett-Heubusch, 1992; Paravicini *et al.*, 1992). Defects in cell wall construction are most often easily revealed by testing for the tendency to undergo cell lysis when treated with glucanases, which digest major components of the cell wall (Levin and Bishop, 1990). Wild-type and $\Delta ptc1 \Delta ptc3$ cells were incubated with β -glucanase and cell lysis was monitored by measuring optical density (Figure 9). The sensitivity to cell wall digestion was found to be similar in both of the strains. As a potential control for this experiment, we also tested an *S.pombe* $\Delta pkc2$ mutant, which has a cell shape defect because it lacks a protein kinase C homolog (Toda *et al.*, 1993). The $\Delta pkc2$ mutant was very sensitive to β -glucanase, strongly indicating that it has a cell wall defect (Figure 9). Direct observation of the cell walls of these strains by thin section electron microscopy provided confirmatory results. The cell walls of wild-type, $\Delta ptc1 \Delta ptc3$ and $\Delta ptc1 \Delta ptc2 \Delta ptc3$ cells were indistinguishable in their thickness and staining, while the $\Delta pkc2$ mutant had a significantly thinner cell wall (data not shown). These results suggest that the *Δptc* mutants have no significant defect in cell wall composition or structure.

Fig. 7. Morphology of wild-type (972), $\Delta ptc1 \Delta ptc3$ (KS920) and $\Delta ptc1 \Delta ptc2 \Delta ptc3$ (KS917) cells. Wild-type and mutant cells were grown at 30°C in YES medium or YES supplemented with 1.2 M sorbitol (+sorb.). Aberrant, swollen cell shape of the double and triple mutants was largely reversed by sorbitol. Bar 10 μ m.

Fig. 8. Temperature-sensitive growth of the $\Delta ptc1 \Delta ptc3$ and $\Delta ptc1 \Delta ptc2 \Delta ptc3$ mutants is suppressed by sorbitol in the medium. Wild-type and (PR109), $\Delta ptc1 \Delta ptc2$ (KS872), $\Delta ptc1 \Delta ptc3$ (KS920), $\Delta ptc2 \Delta ptc3$ (KS890) and $\Delta ptc1 \Delta ptc2 \Delta ptc3$ (KS917) mutant strains were streaked on YES (A and B) or YES + 1.2 M sorbitol (C) agar plates and incubated at 30°C (A) or 36°C (B and C). After 3 days, the plates were photographed.

Mutations in a MAP kinase kinase homolog suppress the $\Delta ptc1 \Delta ptc3$ phenotypes

Having found that $\Delta ptc1 \Delta ptc3$ and $\Delta ptc1 \Delta ptc2 \Delta ptc3$ mutants have normal cell walls, this left an osmoregulation defect as being the most likely cause of the cell shape and cell lysis phenotypes in these strains. To identify the osmosensing signal transduction pathway regulated by PP2C, we undertook a pseudoreversion analysis of the $\Delta ptc1 \Delta ptc3$ double mutant. We anticipated that mutations which suppressed $\Delta ptc1 \Delta ptc3$ phenotypes and which are alleviated by high-osmolarity media might in turn exhibit the opposite phenotype, namely sensitivity to high-osmolarity media. An initial attempt which screened for suppression of the $\Delta ptc1 \Delta ptc3$ phenotype yielded only very weak suppressors which proved to be difficult to analyze, although most acquired a sensitivity to high-salt

Fig. 9. Cell wall digestion of wild-type (PR109), $\Delta ptc1 \Delta ptc3$ (KS920) and $\Delta pck2$ (TP169-1C) strains by glucanase. Cells exponentially growing in YES medium were harvested and incubated with β -glucanase (Zymolyase) at 30°C with vigorous shaking. Cell lysis was monitored by measuring optical density at 600 nm (the value before adding the enzyme was taken as 100%). Wild-type and $\Delta ptc1 \Delta ptc3$ cells showed similar sensitivity to glucanase treatment.

media as predicted (data not shown). While this screen was underway, it was reported that the *Arabidopsis ABII* gene, which is essential for response to the plant hormone abscisic acid, encoded a PP2C protein having an N-terminal extension with sequence homology to EF hand calcium binding sites (Leung *et al.*, 1994; Meyer *et al.*, 1994). This prompted us to perform experiments which led to the discovery that the $\Delta ptc1 \Delta ptc3$ mutant is hypersensitive to CaCl_2 . The $\Delta ptc1 \Delta ptc3$ mutant cells failed to grow in media containing 80 mM CaCl_2 , whilst wild-type cells grew normally (Figure 10B). The $\Delta ptc1 \Delta ptc3$ mutant was also hypersensitive to MnCl_2 , but not to ZnCl_2 (data not shown). Sensitivity to high calcium concentrations was rescued by osmotic stabilizers (data not shown). Therefore, we isolated Ca^{2+} -resistant revertants from the $\Delta ptc1 \Delta ptc3$ mutant. Cells were plated at 30°C on YES medium containing 80 mM CaCl_2 . Since mutations unrelated to the osmosensing pathway could be expected to confer Ca^{2+} resistance (mutations in permeability, cellular Ca^{2+} -metabolism, etc.), Ca^{2+} -resistant colonies were further screened for high-osmolarity sensitivity. By replica plating to YES plates containing 1 M KCl or 1.5 M sorbitol, 16 were found to

Fig. 10. Growth of the $\Delta ptc1 \Delta ptc3$ (KS920), $\Delta wis1$ (JM544) and $\Delta ptc1 \Delta ptc3 \Delta wis1$ (KS1060) strains in the presence of 80 mM CaCl_2 (B) or 1 M KCl (C) at 32°C. Ca^{2+} -sensitivity of $\Delta ptc1 \Delta ptc3$ is rescued by the $\Delta wis1$ mutation. While the $\Delta ptc1 \Delta ptc3$ cells can grow on high-osmolarity medium, the $\Delta wis1$ and $\Delta ptc1 \Delta ptc3 \Delta wis1$ triple mutants show a severe growth defect on the same medium.

show tight high-salt and high-sorbitol sensitivity from ~300 Ca^{2+} -resistant colonies. These strains also exhibited an enhanced *ts* phenotype (data not shown), which possibly explains why we were unable to isolate effective suppressors of the $\Delta ptc1 \Delta ptc3$ *ts* phenotype. Back-crosses with the parental strains and tetrad analysis demonstrated that these revertants carried single mutations which rescued the Ca^{2+} -sensitivity of $\Delta ptc1 \Delta ptc3$ and conferred the high-osmolarity and *ts* growth phenotype. Nine of the 16 revertants contained suppressor mutations that could be out-crossed into a *ptc*⁺ background. Two complementation groups have been identified from them; *spc1* and *spc2* (suppressor of phosphatase 2C) are composed of five and four mutants respectively. These mutations confer a high-osmolarity and *ts* growth phenotype in the *ptc*⁺ background. Eight of them are recessive, but one of the *spc1* mutants (*spc1-M1*) appeared to be dominant, since the heterozygous diploid constructed with wild-type strain showed high-osmolarity sensitivity. The remaining seven have suppressor mutations that are lethal when out-crossed from the $\Delta ptc1 \Delta ptc3$ background. Preliminary results indicate that these identify an additional three complementation groups.

In the budding yeast *S.cerevisiae*, a MAP kinase cascade involved in osmosensing has been identified. Mutations in the *HOG1* MAP kinase and *PBS2* MAP kinase cause growth defects in high-osmolarity medium (Boguslawski, 1992; Brewster *et al.*, 1993). Although it is not clear whether *S.pombe* has an equivalent signal transduction pathway, Wis1 protein kinase, which was originally isolated as a mitotic regulator, shares a significant structural similarity with *PBS2* (Warbrick and Fantes, 1991). In fact, *wis1::ura4*⁺ ($\Delta wis1$) mutant cells

are sensitive to 1 M KCl (Figure 10C) and 1.5 M sorbitol (data not shown). In order to test whether *spc1* or *spc2* mutants are allelic to *wis1*, these mutant strains were crossed to a $\Delta wis1$ mutant. Among 5000 spores that were analyzed in the cross involving *wis1* and *spc2*, no high-salt-resistant recombinants were obtained, showing that *spc2* is allelic to *wis1*. No linkage was seen between *spc1* and *wis1*. To confirm this result, a triple mutant strain, $\Delta ptc1 \Delta ptc3 \Delta wis1$, was constructed. The $\Delta wis1$ mutation suppressed the Ca^{2+} -sensitive phenotype of $\Delta ptc1 \Delta ptc3$ (Figure 10B). Microscopic observation showed that the triple mutant cells have a normal cylindrical shape, indicating that the $\Delta wis1$ mutation also suppresses the aberrant cell morphology of $\Delta ptc1 \Delta ptc3$ (data not shown). This triple mutant showed *ts* growth, since the *wis1* mutant itself also has a *ts* phenotype (data not shown).

These data show that Wis1 is required for osmosensing in fission yeast, probably being a functional equivalent of Pbs2 kinase in *S.cerevisiae*. The data showing that the $\Delta ptc1 \Delta ptc3$ phenotypes can be suppressed by osmotic stabilizers and by inactivation of Wis1 strongly suggest that the PP2C phosphatases encoded by *ptc1*⁺ and *ptc3*⁺ are also involved in the osmoregulation of fission yeast cells, acting in a counteractive fashion to the Wis1 kinase signal transduction pathway.

Discussion

Although type 2C protein phosphatases have long been recognized as a distinct evolutionary class of protein phosphatase, the key physiological functions of PP2C enzymes remain to be discovered. Given the fact that many members of the other classes of protein phosphatases have recently been shown to have crucial roles in a wide range of mechanisms of cellular regulation, we felt that an intensive genetic investigation of PP2C functions was in order. An underlying assumption of these studies was that PP2C enzymes were likely to have very important cellular functions, but that these functions had thus far remained obscure because of overlapping activities of PP2C subtypes encoded by multiple genes.

Emerging PP2C subfamilies

The initial goal of this study was to gain a basic understanding of the number and types of PP2C enzymes that exist in a model eukaryotic organism. With this aim in mind, we have thus far identified three genes encoding PP2C in the fission yeast *S.pombe*. In terms of sequence homologies, it is becoming clear that there are distinct PP2C subfamilies. Among the three proteins discovered in *S.pombe*, Ptc2 and Ptc3 are much more closely related to each other, being ~66% identical in the 260 amino acid core domain, versus ~33% identity to Ptc1. Ptc2 and Ptc3 also share an N-terminal 31 amino acid region which is ~58% identical; this region bears no homology to the longer N-terminal extension of Ptc1 or *S.cerevisiae* PTC1/TPD1 (Maeda *et al.*, 1994; Robinson *et al.*, 1994). Importantly, the N-terminal region of Ptc2 and Ptc3 is also shared with the known mammalian PP2C enzymes, for instance being ~50% identical to the N-termini of rat PP2C α and β isozymes (Tamura *et al.*, 1989; Wenk *et al.*, 1992). Such homologies can be very important. A recent study on *Drosophila* protein phosphatase V (PPV) demon-

strated that the N-terminal 50 residues are crucial for the ability of the PPV gene to complement *sit4* mutations in *S.cerevisiae* and fusion of this domain to the catalytic domain of PP1 can confer this complementation activity (Mann *et al.*, 1993). It is probably also significant that fission yeast Ptc2 and Ptc3 and rat PP2C isozymes also account for the majority of total PP2C activity in their respective organisms. These comparisons clearly demonstrate that PP2C subfamilies can be conserved amongst highly divergent species.

Schizosaccharomyces pombe Ptc1 shows higher homology to the PP2C encoded by *S.cerevisiae* PTC1/TPD1 (45% identity in the central domain) than to Ptc2 or Ptc3. Therefore, *S.pombe* *ptc1*⁺ and *S.cerevisiae* PTC1/TPD1 might compose another subfamily of PP2C genes. However, functional differences have been found between these two genes. Mutations in budding yeast PTC1/TPD1 cause a *ts* growth phenotype accompanied by a deficiency in tRNA splicing (Maeda *et al.*, 1993; Robinson *et al.*, 1994). The *S.pombe* *ptc1* deletion mutant can grow well at high temperatures, although it is more sensitive to exposure to 48°C than is the wild-type (Shiozaki *et al.*, 1994). *Schizosaccharomyces pombe* might have another PTC1/TPD1-like phosphatase which has not been identified. In fact, a significant amount of PP2C activity was detected in the Δ *ptc1* Δ *ptc2* Δ *ptc3* triple mutant extracts, confirming the existence of at least one additional PP2C gene in *S.pombe*.

Cellular functions of PP2C homologs in *S.pombe*

Having identified three *ptc* genes accounting for ~90% of total PP2C activity in *S.pombe* cell extracts, the next major goal was to characterize cellular defects arising from loss of PP2C activity. As mentioned before, the *ptc1*⁺ gene was first isolated as a multicopy suppressor of *swol-26*, a mutation of an hsp90 gene in *S.pombe*. We found that *ptc1*⁺ mRNA expression increases in response to heat shock and Δ *ptc1* mutant cells are hypersensitive to heat shock (Shiozaki *et al.*, 1994). In contrast, expression of neither *ptc2*⁺ nor *ptc3*⁺ is induced by heat shock and Δ *ptc2* and Δ *ptc3* mutant cells are not heat shock-sensitive (data not shown). These observations suggest a unique physiological role of the PP2C subtype encoded by *ptc1*⁺. On the other hand, *ptc1*⁺ also has an overlapping function with *ptc2*⁺ and *ptc3*⁺ in maintaining osmotic stability. The Δ *ptc1* Δ *ptc3* and Δ *ptc1* Δ *ptc2* Δ *ptc3* mutant cells are abnormally swollen and they lyse at elevated temperatures. These phenotypes are suppressed by osmotic stabilizers. We considered the possibility that these phenotypes result from aberrant cell wall construction, since it was previously shown that *S.pombe* mutants defective in cell wall glucan synthesis are swollen and tend to lyse at elevated temperatures and that this phenotype is rescued by adding osmotic stabilizers to growth media (Ribas *et al.*, 1991; Díaz *et al.*, 1993). Moreover, deletion of the *kin1*⁺ protein kinase causes a growth polarity defect which is accompanied by altered sensitivity to glucanase digestion (Levin and Bishop, 1990). However, we were unable to detect any cell wall defect in the Δ *ptc1* Δ *ptc3* and Δ *ptc1* Δ *ptc2* Δ *ptc3* mutants, while as a positive control for these experiments we showed that the aberrant cell shape phenotype of the protein kinase C mutant Δ *pkc2* (Toda *et al.*, 1993) could be explained by the fact that the

cell wall of this mutant is thinner and more sensitive to glucanase digestion.

We next considered that the phenotypes of the Δ *ptc1* Δ *ptc3* and Δ *ptc1* Δ *ptc2* Δ *ptc3* mutants, particularly the tendency to undergo cell lysis that is suppressed by increasing the osmolarity of the medium, might be caused by an osmoregulation defect. It is known, for example, that the budding yeast *S.cerevisiae* responds to increases in extracellular osmolarity by accumulating cytoplasmic glycerol (Blomberg and Adler, 1989). A MAP kinase cascade is involved in this response. Mutants in the *HOG1* MAP kinase and *PBS2* MAP kinase kinase display growth defects in high-osmolarity media (Boguslawski, 1992; Brewster *et al.*, 1993). If a similar pathway existed in *S.pombe*, one might expect that hyperactivation of that pathway would lead to precisely the same phenotype as observed in the Δ *ptc1* Δ *ptc3* mutant. With such a possibility in mind, we undertook a Δ *ptc1* Δ *ptc3* pseudoreversion screen, specifically targeting mutants that acquired hypersensitivity to high-osmolarity media. Having first encountered no success with suppressing the Δ *ptc1* Δ *ptc3* *ts* phenotype, we subsequently discovered that Δ *ptc1* Δ *ptc3* mutants were acutely sensitive to Ca²⁺. Having then discovered mutations that effectively suppressed the Ca²⁺-sensitive phenotype and simultaneously caused sensitivity to high-osmolarity conditions, it then became abundantly clear why the first pseudoreversion screen had failed. The suppressor mutations, for example of *spc1* and *spc2*, independently caused a *ts* lethal phenotype.

Genetic analysis established that *spc2*⁺ was allelic to *wis1*⁺, which encodes a MAP kinase kinase homolog. The *wis1*⁺ gene was originally isolated as a dosage-dependent inducer of mitosis, but its primary structure is most similar to budding yeast *PBS2* (Warbrick and Fantes, 1991). In fact, as we report here, *wis1/spc2* mutant cells show a severe growth defect in high-osmolarity medium, apparently equivalent to the phenotype of *S.cerevisiae* *pbs2* mutants. Similar findings have recently been reported by Fantes and colleagues (Stettler *et al.*, manuscript submitted). From this we conclude that *S.pombe*, like *S.cerevisiae*, has a MAP kinase signal transduction cascade which is required for osmoregulation. We have not yet tested whether *wis1*⁺ and *PBS2* are functionally interchangeable, but we think that it is likely to be the case.

Recently, Maeda *et al.* identified *S.cerevisiae* PP2C gene homologs, PTC1 and PTC3, and a tyrosine phosphatase gene, PTP2, as multicopy suppressors of mutations in the *SLN1* gene, which encodes a protein homologous to the histidine kinases and regulator proteins in bacterial two-component systems (Maeda *et al.*, 1994). Their results suggest that *SLN1* regulates the *PBS2*–*HOG1* MAP kinase cascade through a regulator protein encoded by *SSK1* and that PTC1, PTC3 and PTP2 phosphatases could be negative regulators of this MAP kinase cascade or possibly regulate upstream or downstream components of the signal transduction pathway. These inferences on the functions of the PTC1, PTC3 and PTP2 phosphatases were based entirely on over-production phenotypes. If this proposal was correct, then one would expect that mutations causing loss of one or more of these phosphatases would lead to hyperactivation of the osmosensing pathway. This would result in an increase in intracellular osmolarity that could be lethal if it were not counteracted by an increase in the

Table II. *S.pombe* strains used in this study

Strains	Genotype	Source or reference
972	<i>h⁻</i>	our stock
PR109	<i>h⁻ leu1-32 ura4-D18</i>	our stock
PR166	<i>h⁻ leu1-32 ura4-D18 swol-26</i>	Aligue <i>et al.</i> (1994)
CHP428	<i>h⁺ leu1-32 ura4-D18 his7-366 ade6-M210</i>	C.S.Hoffman
CHP429	<i>h⁻ leu1-32 ura4-D18 his7-366 ade6-M216</i>	C.S.Hoffman
KS834	<i>h⁻ leu1-32 ura4-D18 ptc1::LEU2</i>	Shiozaki <i>et al.</i> (1994)
KS858	<i>h⁻ leu1-32 ura4-D18 ptc2::ura4⁺</i>	this study
KS948	<i>h⁻ leu1-32 ura4-D18 ptc3::ura4⁺</i>	this study
KS872	<i>h⁻ leu1-32 ura4-D18 ptc1::LEU2 ptc2::ura4⁺</i>	this study
KS890	<i>h⁻ leu1-32 ura4-D18 ptc2::ura4⁺ ptc3::ura4⁺</i>	this study
KS917	<i>h⁻ leu1-32 ura4-D18 ptc1::LEU2 ptc2::ura4⁺ ptc3::ura4⁺</i>	this study
KS920	<i>h⁺ leu1-32 ura4-D18 ptc1::LEU2 ptc3::ura4⁺</i>	this study
KS1019	<i>h⁻ leu1-32 ura4-D18 his7-366 ade6-M210 ptc1::LEU2 ptc3::his7⁺</i>	this study
KS1048	<i>h⁺ leu1-32 ura4-D18 his7-366 ade6-M216 ptc1::LEU2 ptc3::his7⁺</i>	this study
JM544	<i>h⁻ leu1-32 ura4-D18 wis1::ura4⁺</i>	J.B.A.Millar
KS1060	<i>h⁻ leu1-32 ura4-D18 his7-366 ptc1::LEU2 ptc3::his7⁺ wis1::ura4⁺</i>	this study
TP169-1C	<i>h⁻ leu1-32 ura4-D18 pck2::LEU2</i>	Toda <i>et al.</i> (1993)
RM13	<i>h⁻ leu1-32 ura4-D18 his7-366 ade6-M210 ptc1::LEU2 ptc3::his7⁺ spc1-M13</i>	this study
RP10	<i>h⁺ leu1-32 ura4-D18 his7-366 ade6-M216 ptc1::LEU2 ptc3::his7⁺ spc2-P10</i>	this study

osmolarity of the growth medium. In other words, the cells could be expected to swell and perhaps then burst in medium that was not osmotically stabilized. As reported here, this is precisely the phenotype which we have observed in $\Delta ptc1 \Delta ptc3$ and $\Delta ptc1 \Delta ptc2 \Delta ptc3$ mutants. Moreover, as mentioned above, this phenotype can be effectively suppressed by inactivation of Wis1 MAP kinase kinase. Thus, our results substantiate the idea that PP2C enzymes have as part of their regular function the responsibility for counteracting the osmosensing signal which is transduced by a MAP kinase pathway.

We have not yet attempted to identify the substrate of PP2C in the fission yeast osmosensing pathway; indeed, it is likely to be a complicated pathway of which we currently know only one participant, Wis1. By analogy with other MAP kinase cascades, we can be assured that at least three components of the pathway are positively regulated by serine/threonine phosphorylation: Wis1, the MAP kinase(s) phosphorylated by Wis1 and the substrate(s) of that MAP kinase. In addition, studies of other MAP kinase pathways suggest that there are likely to be other kinases functioning upstream of Wis1. Isolation of the gene encoding the MAP kinase will provide an important tool for this analysis, since activation of this kinase will involve tyrosine phosphorylation and thus can be easily monitored by the use of anti-phosphotyrosine antibodies. The *spc1* mutants which we have isolated behave identically to the *wis1/spc2* mutants, therefore there is a good chance that *spc1⁺* encodes either the MAP kinase acting downstream of Wis1 or the MAP kinase kinase acting upstream. In addition to *spc1* and *wis1/spc2*, we have tentatively identified three other complementation groups (*spc3*, *spc4* and *spc5*) which have similar properties, except that they are lethal when outcrossed from the $\Delta ptc1 \Delta ptc3$ background. Our assumption is that mutations of these genes cause an osmosensing defect which is more severe than *spc1* or *wis1/spc2* mutations, therefore they are also likely to identify components of the same signal transduction pathway. Experiments are currently underway to clone *spc1⁺*, *spc3⁺*, *spc4⁺* and *spc5⁺*.

Over the last few years it has become abundantly clear

that MAP kinase cascades have crucial roles in a number of signal transduction pathways, governing cell proliferation, differentiation and various stress responses in all eukaryotic organisms. Elements that transmit signals upstream and downstream of the MAP kinase cascades are not necessarily homologous. Therefore, since we do not yet know which proteins are targeted for dephosphorylation by PP2C, it would be premature to predict that PP2C enzymes are likely to have important roles in regulating all processes that are modulated by MAP kinase cascades. However, since even yeasts have multiple MAP kinase cascades, evaluation of such a possibility can be carried out using a genetically tractable model organism.

Materials and methods

Yeast strains and media

Schizosaccharomyces pombe strains used in this study are listed in Table II. They are derivatives of 972^{h⁻} and 975^{h⁺} (Mitchison, 1970). Standard procedures for *S.pombe* genetics were followed according to Gutz *et al.* (1974) and Moreno *et al.* (1991). YES and synthetic EMM2 media were used in growing *S.pombe* cells.

Isolation of *ptc2⁺* and *ptc3⁺* genes

Degenerate oligonucleotides used for PCR were: PPC52 (5' primer) 5'-GCIGTTT(A/T)(C/T)GA(C/T)GGICA-3' and PPC31 (3' primer) 5'-AC(A/G)TCCCAIA(A/G/T)ICC(A/G)TC(A/G)CA-3' (I, inosine). *Schizosaccharomyces pombe* genomic DNA was isolated from the *ptc1* deletion strain (Shiozaki *et al.*, 1994) and used as a template. PCR amplification was performed with *Taq* DNA polymerase (Gibco/BRL) by 40 cycles at 95°C for 1 min, 37°C for 1 min, 72°C for 1 min. PCR products (0.5 kb, *ptc2⁺*; 0.8 kb, *ptc3⁺*) were cloned into the pCRII vector (Invitrogen) following the instructions from the supplier. DNA sequencing and genomic Southern analysis revealed that both of the cloned fragments are derived from the *S.pombe* chromosomal genes encoding new PP2C homologs (see Results). Using the cloned fragments as probes, a *S.pombe* genomic library in the pFL20 vector (Heyer *et al.*, 1986) was screened by hybridization to isolate genomic DNA fragments containing entire genes. One clone (pFL20-18) for *ptc2⁺* and two clones (pFL20-431 and pFL20-1512) for *ptc3⁺* were isolated and the open reading frames (ORF) were localized by restriction mapping and DNA sequencing. Procedures described by Sambrook *et al.* (1989) were followed for general nucleic acid manipulation.

Gene disruption

The one-step gene disruption method (Rothstein, 1983) was employed to construct the *ptc2* and *ptc3* deletion strains. For *ptc2* disruption, a

2.9 kb *Bgl*II–*Bam*HI fragment of the *ptc2*⁺ genomic clone was subcloned into the *Bam*HI site of vector pUC19 and the *Cla*I–*Sac*I 0.7 kb region of *ptc2*⁺ was substituted with a 1.8 kb *Hind*III fragment of the *S.pombe ura4*⁺ gene. The resulting plasmid was digested with *Eco*RI and *Sall* to liberate the *ptc2::ura4*⁺ fragment and used for the transformation of a diploid *h⁻lh⁺ leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216*. For disrupting *ptc3*⁺, a *ptc3⁺ Hind*III 3.7 kb fragment cloned in pBluescript (Stratagene) was digested with *Bam*HI and a 1.8 kb *Hind*III fragment of *ura4*⁺ was inserted. The *Hind*III 4.7 kb fragment containing *ptc3::ura4*⁺ was used for transformation of the diploid strain described above. Stable Ura⁺ transformants were selected and gene disruption was confirmed by Southern hybridization. After sporulation, phenotypes of the haploid segregants were analyzed.

Phosphatase assay

Fractionation of *S.pombe* crude lysates by MonoQ chromatography was performed as described by Shiozaki *et al.* (1994). Preparation of ³²P-labeled casein and the procedures of the PP2C phosphatase assay were as described by McGowan and Cohen (1988). Okadaic acid (100 nM; Calbiochem) was used to inhibit PP2A activity in the crude extracts.

Glucanase sensitivity experiments

Glucanase sensitivity of *ptc* mutants was evaluated following the procedures described by Levin and Bishop (1990). Wild-type (PR109), Δ *ptc1* Δ *ptc3* (KS920) and Δ *pc2* (TP169-1C) strains were grown to mid-logarithmic phase in YES medium at 30°C. The cells were harvested, washed once with TE buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 1 mM 2-mercaptoethanol) and inoculated into the same buffer containing either 25 µg/ml α -glucanase (Novozyme 234, Novoindustry) or 100 µg/ml β -glucanase (Zymolyase-20T; Seikagaku Corporation). Cell suspensions were incubated at 30°C with vigorous shaking and cell lysis was monitored by measuring optical density at 600 nm.

Isolation of suppressor mutations of Δ *ptc1* Δ *ptc3*

The Δ *ptc1* Δ *ptc3* strains KS1019 and KS1048 were grown in rich YES medium to saturation. Three separate cultures were prepared for each strain. About 10⁷ cells were plated on a YES agar plate supplemented with 80 mM CaCl₂, using four plates for each of the cultures. After incubation at 32°C for 4–5 days, Ca²⁺-resistant colonies were tested for their high-osmolarity sensitivity by replica plating onto YES agar plates containing 1 M KCl and 20 mg/l Phloxin B dye (Sigma). Sixteen Ca²⁺-resistant, high-salt-sensitive colonies were picked up. Two of these were found to be sterile and only the remaining 14 were subjected to further analysis. An allelism test was performed by crossing the revertants from KS1019 with the revertants from KS1048 and high-salt sensitivity and Ca²⁺ resistance of the segregants were examined. Five *spc1* mutants (*M1*, *M8*, *M13*, *P13* and *P15*) and four *spc2* (allelic to *wis1*) mutants (*M14*, *M16*, *M76* and *P10*) were also crossed with the parental strain of opposite mating type and the resultant diploids were examined for resistance to 1 M KCl. Only *spc1-M1* showed a dominant high-salt-sensitive phenotype, while the other eight mutations were found to be recessive.

Acknowledgements

We are grateful to Dr C.-M.Chang for his skilful help in electron microscopy and Drs T.Toda, J.B.A.Millar and C.S.Hoffman for strains. We also thank Dr C.H.McGowan for critical reading of the manuscript. K.S. is a recipient of a long-term fellowship from the Human Frontier Science Program. This research was supported by NIH grant GM-41281 awarded to P.R.

References

- Aligue,R., Akhavan-Niaki,H. and Russell,P. (1994) *EMBO J.*, **13**, 6099–6106.
- Blomberg,A. and Adler,L. (1989) *J. Bacteriol.*, **171**, 1087–1092.
- Boguslawski,G. (1992) *J. Gen. Microbiol.*, **138**, 2425–2432.
- Brewster,J.L., de Valoir,T., Dwyer,N.D., Winter,E. and Gustin,M.C. (1993) *Science*, **259**, 1760–1763.
- Burns,J.M., Parsons,M., Rosman,D.E. and Reed,S.G. (1993) *J. Biol. Chem.*, **268**, 17155–17161.
- Cohen,P. (1989) *Annu. Rev. Biochem.*, **58**, 453–508.
- Cohen,P. and Cohen,P.T.W. (1989) *J. Biol. Chem.*, **264**, 21435–21438.
- Díaz,M., Sanchez,Y., Bennett,T., Sun,C.R., Godoy,C., Tamanai,F., Duran,A. and Perez,P. (1993) *EMBO J.*, **12**, 5245–5254.
- Gutz,H., Heslot,H., Leupold,U. and Lopreno,N. (1974) In King,R.C. (ed.), *Handbook of Genetics*. Plenum Press, New York, pp.395–446.
- Heyer,W.-D., Sipiczki,M. and Kohli,J. (1986) *Mol. Cell. Biol.*, **6**, 80–89.
- Kinoshita,N., Ohkura,H. and Yanagida,M. (1990) *Cell*, **63**, 405–415.
- Lawson,J.E., Niu,X.-D., Browning,K.S., Trong,H.L., Yan,J. and Reed,L.J. (1993) *Biochemistry*, **32**, 8987–8993.
- Leung,J., Bouvier-Durand,M., Morris,P.-C., Guerrier,D., Chefedor,F. and Giraudat,J. (1994) *Science*, **264**, 1448–1452.
- Levin,D.E. and Bartlett-Heubusch,E. (1992) *J. Cell Biol.*, **116**, 1221–1229.
- Levin,D.E. and Bishop,J.M. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 8272–8276.
- Levin,D.E., Fields,F.O., Kunisawa,R., Bishop,J.M. and Thorner,J. (1990) *Cell*, **62**, 213–224.
- Maeda,T., Tsai,A.Y.M. and Saito,H. (1993) *Mol. Cell. Biol.*, **13**, 5408–5417.
- Maeda,T., Wurgler-Murphy,S.M. and Saito,H. (1994) *Nature*, **369**, 242–245.
- Mann,D.J., Campbell,D.G., McGowan,C.H. and Cohen,P.T.W. (1992) *Biochim. Biophys. Acta*, **1130**, 100–104.
- Mann,D.J., Dombrádi,V. and Cohen,P.T.W. (1993) *EMBO J.*, **12**, 4833–4842.
- McGowan,C.H. and Cohen,P. (1988) *Methods Enzymol.*, **159**, 416–426.
- Meyer,K., Leube,M.P. and Grill,E. (1994) *Science*, **264**, 1452–1455.
- Mitchison,J.M. (1970) *Methods Cell Physiol.*, **4**, 131–146.
- Moreno,S., Klar,A. and Nurse,P. (1991) *Methods Enzymol.*, **194**, 795–823.
- Mumby,M.C. and Walter,G. (1993) *Physiol. Rev.*, **73**, 673–699.
- Ota,I.M. and Varshavsky,A. (1993) *Science*, **262**, 566–569.
- Paravicini,G., Cooper,M., Friedli,L., Smith,D.J., Carpentier,J.-L., Klig,L.S. and Payton,M.A. (1992) *Mol. Cell. Biol.*, **12**, 4896–4905.
- Prabhala,G., Rosenberg,G.H. and Käufer,N.F. (1992) *Yeast*, **8**, 171–182.
- Pratt,M.L., Maher,J.F. and Roche,T.E. (1982) *Eur. J. Biochem.*, **125**, 349–355.
- Ribas,J.C., Díaz,M., Duran,A. and Perez,P. (1991) *J. Bacteriol.*, **173**, 3456–3462.
- Robinson,M.K., van Zyl,W.H., Phizicky,E.M. and Broach,J.R. (1994) *Mol. Cell. Biol.*, **14**, 3634–3645.
- Rothstein,R.J. (1983) *Methods Enzymol.*, **101**, 202–211.
- Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shiozaki,K., Akahavan-Niaki,H., McGowan,C.H. and Russell,P. (1994) *Mol. Cell. Biol.*, **14**, 3742–3751.
- Tamura,S., Lynch,K.R., Lerner,J., Fox,J., Yasui,A., Kikuchi,K., Suzuki,Y. and Tsuiki,S. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 1796–1800.
- Teague,W.M., Pettit,F.H., Wu,T.-L., Silberman,S.R. and Reed,L.J. (1982) *Biochemistry*, **21**, 5585–5592.
- Toda,T., Shimanuki,M. and Yanagida, M. (1993) *EMBO J.*, **12**, 1987–1995.
- Warbrick,E. and Fantes,P.A. (1991) *EMBO J.*, **10**, 4291–4299.
- Wenk,J., Trompeter,H.-I., Pettrich,K.-G., Cohen,P.T.W., Campbell,D.G. and Mieskes,G. (1992) *FEBS Lett.*, **297**, 135–138.

Received on August 1, 1994; revised on November 21, 1994