Protein Phosphatase 2C Acts Independently of Stress-activated Kinase Cascade to Regulate the Stress Response in Fission Yeast*

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Stress-activated signal transduction pathways, which are largely conserved among a broad spectrum of eukaryotic species, have a crucial role in the survival of many forms of stress. It is therefore important to discover how these pathways are both positively and negatively regulated. Recent genetic studies have implicated protein phosphatase 2C (PP2C) as a novel negative regulator of stress response pathways in both budding and fission yeasts. Moreover, it was hypothesized that PP2C dephosphorylates one or more components of protein kinase cascades that are at the core of stress-activated signal transduction pathways. Herein we present genetic and biochemical studies of the fission yeast Schizosaccharomyces pombe that disprove this hypothesis and indicate that PP2C instead negatively regulates a downstream element of the pathway. First, high expression of PP2C produces phenotypes that are inconsistent with negative regulation of the Wtk1-Wis1-Spc1 stress-activated kinase cascade. Second, high expression of PP2C leads to sustained activating tyrosine phosphorylation of Spc1. Third, Spc1-dependent phosphorylation of Atf1, a transcription factor substrate of Spc1, is unaffected by high expression of PP2C. Fourth, high expression of PP2C suppresses Atf1-dependent transcription of a stress-response gene. These studies strongly suggest that PP2C acts downstream of Spc1 kinase in the stress-activated signal transduction pathway.

Eukaryotic organisms frequently encounter environmental conditions that cause cytotoxic damage; hence, they have developed sophisticated systems of sensing and responding to physiological stress. Protein kinase cascades are at the core of these stress sensor pathways (1). These cascades follow the paradigm established for mitogen-activated protein kinase (MAPK)1 cascades: a MAPK kinase kinase (MAPKKK) phosphorylates a MAPK kinase (MAPKK), which in turn phosphorylates a MAPK. The MAPKK and MAPK components are serine-threonine kinases, whereas MAPKKs are dual specificity enzymes, activating MAPK substrates by phosphorylating threonine and tyrosine residues in a conserved motif (2). It is not understood why protein kinase cascades are used to transmit stress signals, although it is likely that the spatial distribution of the cascade elements facilitates rapid signaling from the cell surface to the nucleus where MAPK homologs phosphorylate transcription factor substrates.

Recent studies have revealed impressive functional and structural conservation of stress response pathways in yeast, plants, and various metazoan species, including humans (3). The fission yeast Schizosaccharomyces pombe has been a focus of some of the most interesting investigations, in part because studies of fission yeast have uncovered a link between stress response pathways and cell cycle control (4, 5). The S. pombe stress-activated kinase cascade consists of Wtk1-Wis1-Spc1 kinases (6). The Spc1 MAPK homolog, which is also known as Sty1 and Phh1 (4, 7), is highly similar to mammalian p38 kinases (8) and Hog1p kinase of the budding yeast Saccharomyces cerevisiae (9). Like p38 kinase, Spc1 is broadly responsive to many forms of stress, including high osmolarity, oxidative stress, UV irradiation, and heat stress, as well as carbon and nitrogen starvation (4, 5, 10, 11). Recent investigations have further revealed that mammalian p38 and fission yeast Spc1 share similar substrates. In the case of Spc1, the substrate is Atf1, a transcription factor containing a bZIP (basic leucine zipper) domain. Remarkably, Atf1 is highly similar to ATF-2, a mammalian transcription factor that is widely believed to be an important substrate of p38 kinase in vivo (11, 12).

It is evident that fission and budding yeasts can serve as useful model systems for uncovering novel modes of regulation of stress-activated protein kinase cascades. This accounts for the interest in recent studies suggesting that stress-activated kinase cascades in yeast are negatively regulated by two types of protein phosphatases: tyrosine-specific enzymes (4, 5, 10) and serine-threonine phosphatases of the type 2C class (PP2C) (5, 13). Evidence for a role of tyrosine-specific phosphatases in the negative regulation of stress-activated kinase cascades first arose from genetic studies of S. cerevisiae. The gene PTP2 was cloned as a high copy suppressor of mutations that cause lethality due to hyperactivation of the Hog1p kinase cascade. High expression of PTP2 results in decreased tyrosine phosphorylation of Hog1p, a finding consistent with the conclusion that Ptp2p directly dephosphorylates Hog1p in vivo (13). Ptp2p is assumed to be a tyrosine-specific enzyme because it is more closely related to tyrosine-specific phosphatases than to dual specificity tyrosine/threonine phosphatases that have been identified as MAPK phosphatases in mammalian cells, although the enzymatic specificity of Ptp2p has not been examined. In the case of S. pombe, a critical advance was made with

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAP kinase; MAPKKK, MAP kinase kinase; PP2C, protein phosphatase 2C; EMM2, Edinburgh minimal medium; NiCl2, nickel chloride; NTA, Ni2+–nitrilotriacetic acid; HA, hemagglutinin; Ha6H, hemagglutinin antigen epitope and six histidines; PTP, protein-tyrosine phosphatase.
the discovery that the synthetic lethal phenotype observed in a strain lacking two tyrosine phosphatase genes, pyp1 and pyp2, was effectively suppressed by null mutations in genes encoding elements of the Wis1-Spc1 kinase cascade. Moreover, the stress-sensitive and G2 cell cycle delay phenotypes exhibited by spc1 and wis1 mutants were replicated by high expression of Pyp1 and Pyp2 enzymes (4, 5). These genetic studies were followed by definitive biochemical findings showing that Pyp1 and Pyp2 directly dephosphorylated tyrosine 173 of Spc1 both in vivo and in vitro (5, 10). Interestingly, pyp2 expression is induced in response to stress by a process that requires the Wis1-Spc1 kinase cascade and Atf1 transcription factor, indicating a mechanism of negative feedback regulation of the Wis1-Wis1-Spc1 signal transduction pathway (11, 12).

In contrast to the situation with tyrosine-specific phosphatases, the role of PP2C in the negative regulation of stress-activated kinase cascades is poorly understood. The initial insights came from studies of S. cerevisiae that revealed that two PP2C genes, PTC1 and PTC3, were multicyclic suppressors of mutations that caused hyperactivation of the Hog1p kinase cascade (13). The suggestion that PP2C negatively regulates the Hog1p stress-activated kinase cascade was further supported by the discovery of a synthetic lethal interaction involving ptp2 and ptc1 mutations, which was suppressed by hog1 mutations (14). These findings led to the hypothesis that PP2C negatively regulates the stress signaling pathway by dephosphorylating Hog1p or Pbs2p, the MAPKK homolog that negatively regulates the stress signaling pathway by dephosphorylation of Spc1 kinase cascade (13). The suggestion that PP2C negatively regulates Hog1p was effectively supported by the discovery that the synthetic lethal phenotype observed in a strain lacking two tyrosine phosphatase genes, pyp1 and pyp2 (15, 16). These defects are accounted for by mutations in PTC1, PTC2, and PTC3, which encode a PP2C enzyme that is required to interact with Hog1p. Independent evidence for a role of PP2C in the negative regulation of stress-activated kinase cascades came from studies of S. pombe. In S. pombe the genes ptc1, ptc2, and ptc3 account for ~90% of the total PP2C activity (15, 16).

Mutants carrying deletions of two or three of these genes are hypersensitive to stress-induced transcription of several genes (5, 10). The expression of ptc1, ptc2, and ptc3 mRNA have been described (10, 11). Notably, the expression of ptc1 is driven by the strong constitutive adh1 promoter. Cells were grown to midlog phase at 30 °C, stressed by adjusting the medium to 0.6 M KCl, and harvested by filtration. Quantification of signals was performed using a Molecular Dynamics PhosphorImager.

**Regulation of Stress Response by PP2C**

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Media**—S. pombe PR109 (h’/leu1-32 ura4-D18, KS1146 (h’/leu1-32 ura4-D18 spc1-M13), KS1376 (h’/leu1-32 ura4-D18 spc1-HAH(ura4)+), KS1479 (h’/leu1-32 ura4-D18 spc1-HAH(ura4)+)) and KS1497 (h’/leu1-32 ura4-D18 atf1-HAH(ura4)+) and KS554 (h’/leu1 ura4 wis1:ura4) have been described elsewhere (5, 11). KS1017 (h’/leu1-32 ura4-D18 his1-366 ptc1::LEU2 ptc3::his7), KS1112 (h’/leu1-32 ura4-D18 ptc1::LEU2 pyp1::ura4), KS980 (h’/leu1-32 ura4-D18 ptc1::LEU2 ptc3::ura4), KS1136 (h’/leu1-32 ura4-D18 his1-366 ptc1::LEU2 ptc3::his7), KS1151 (h’/leu1-32 ura4-D18 his1-366 ptc1::LEU2 ptc3::his7 pyp1::ura4 wis1::ura4), and FG1761 (h’/leu1-32 ura4-D18 ptc1::LEU2 ptc3::his7 spc1-HAH(ura4)+) were constructed during the course of these experiments. Yeast extract minimal YED (yeast extract and glucose) and synthetic minimal medium EMM2 were used for growth media. Growth media and fission yeast genetic and biochemical experimental methods have been described elsewhere (22, 23).

**RNA Isolation and Hybridization**—RNA isolation and detection of leu1, pyp2, and gpd1 mRNA have been described (10). NdeI-NdeI fragments from the prEP1-pts1HA6H, prEP1-pts2HA6H, or prEP1-pts3HA6H plasmids were used to probe ptc1, ptc2, or ptc3 mRNA. Cells from various strains were grown to midlog phase at 30 °C, stressed by adjusting the medium to 0.6 M KCl, and harvested by filtration. Quantification of signals was performed using a Molecular Dynamics PhosphorImager.

**FEM-2**—The first aim of our work was to expand the genetic studies implicating PP2C in the stress-sensitive and G2 cell cycle delay phenotypes exhibited by spc1 and wis1 mutants were replicated by high expression of Pyp1 and Pyp2 enzymes (4, 5). These genetic studies were followed by definitive biochemical findings showing that Pyp1 and Pyp2 directly dephosphorylated tyrosine 173 of Spc1 both in vivo and in vitro (5, 10). Interestingly, pyp2 expression is induced in response to stress by a process that requires the Wis1-Spc1 kinase cascade and Atf1 transcription factor, indicating a mechanism of negative feedback regulation of the Wis1-Wis1-Spc1 signal transduction pathway (11, 12).

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Mutants carrying deletions of two or three of these genes are hypersensitive to increased levels of calcium. These defects are accounted for by mutations in PTC1, PTC2, and PTC3, which encode a PP2C enzyme that is required to interact with Hog1p. Independent evidence for a role of PP2C in the negative regulation of stress-activated kinase cascades came from studies of S. pombe. In S. pombe the genes ptc1, ptc2, and ptc3 account for ~90% of the total PP2C activity (15, 16).

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The Δptc1 Δptc3 Δwis1 mutant. This quadruple mutant grew much better than the Δpyp1 Δptc1 Δptc3 triple mutant (Fig. 1), indicating that inactivation of the Wis1-Spc1 kinase cascade suppresses the Δpyp1 Δptc1 Δptc3 triple mutant phenotype. These findings are most simply interpreted to suggest that the Ptc1 and Ptc3 enzymes contribute to the negative regulation of the stress-activated signal transduction pathway.

**ptc1** mRNA is responsive to other forms of stress that are known to activate Spc1 kinase, such as high osmolarity, and whether the activation of Spc1 and increased expression of **ptc1** are causally related. Fig. 2A shows that **ptc1** mRNA increases in response to exposure to high salt media (0.6 M KCl). Maximal **ptc1** expression was reached within 30–40 minutes of exposure to high osmolarity conditions. Induction of **ptc1** expression was completely abolished in spc1-M13 cells (Fig. 2A). Thus, **ptc1** expression is elevated in response to osmotic stress by a Spc1-dependent mechanism.

Atf1 protein, a bZIP transcription factor that is a substrate of Spc1 kinase, shows that high osmolarity stress induces spc1-M13 cells (Fig. 2A). Therefore, of the three known PP2C genes in *S. cerevisiae* have led others to suggest that PP2C might directly dephosphorylate stress-activated kinases. Thus, one hypothesis that explains our genetic findings is that Ptc1 negatively regulates Spc1 by dephosphorylating Spc1, Wis1, or another upstream element of the signal transduction cascade that is activated by serine or threonine phosphorylation, as observed for the known Spc1 negative regulators, Pyp1 and Pyp2 tyrosine phosphatases (5, 11, 12).

Mutants defective for Spc1 or Wis1 activity display characteristic phenotypes, including a delay of the onset of mitosis. Thus, spc1 and wis1 cells divide at a cell length of approximately 20 μm in EMM2 medium, whereas wild type cells divide at approximately 14 μm (5). High expression of pyp1 or pyp2 genes produces an identical cell elongation phenotype, a finding consistent with the demonstration that Pyp1 and Pyp2 inactivate Spc1 kinase via direct dephosphorylation of tyrosine 173 (4, 5, 10). If PP2C enzymes dephosphorylate and thereby inactivate stress-activated protein kinases, then high expression of PP2C should induce phenotypes that are very similar to those observed in spc1 and wis1 mutants as well as Pyp1 and Pyp2 overproducer strains. We therefore examined the cell size phenotype of strains that express **ptc1** at a high level. We employed a strain transformed with a plasmid (pART1-**ptc1** plasmid) expressing **ptc1** under the control of the strong *adh1* promoter (25). Assays of Mono Q chromatographic fractionation samples from extracts of cells transformed with pART1-**ptc1** confirmed that there was a large increase in PP2C activity (Fig. 3A). The increased activity was predominantly detected in fractions 6–8, which corresponds to the chromatography samples from extracts of cells transformed with pART1-**ptc1**.
agar medium containing thiamine, which repressed the nmt promoter for 48 h at 30 °C. Bar, 20 μm.

Expression was induced by plating cells on EMM2 agar lacking thiamine and grown 48 h at 30 °C. For transformed results in a large increase in PP2C activity. PP2C assays of transformant cells were transformed with plasmids expressing those genes (pART1-pto1 and pREP1-pto2). Mono Q chromatographic fractionation samples from extracts of cells transformed with pART1-pto1 were incubated for the indicated times in the presence of 0.6 M KCl. Spc1 protein was precipitated with Ni2+-NTA beads and probed by Western blotting for the presence of phosphotyrosine (α-pTyr). Immunoblot analysis with an anti-HA antibody confirmed that the Spc1 levels were approximately constant throughout the experiment.

A Ptcl overproducer strain. Plasmid pART1-pto1 was transformed into a strain in which the chromosomal copy of spc1 was tagged with a sequence encoding the hemagglutinin antigen epitope and six histidines (Ha6H), allowing easy purification of Spc1 with Ni2+-NTA-agarose beads and detection with anti-HA antibodies. Exposure of cells transformed with a control plasmid (pART1) to osmotic stress, performed in this experiment by suspension of cells in growth medium containing 0.6 M KCl, led to a rapid and transient increase in Spc1 tyrosine phosphorylation (Fig. 4). Spc1 tyrosine phosphorylation reached a maximum level approximately 10 min after the exposure to osmotic stress, a finding that is consistent with previous studies. As shown in Fig. 4, exposure of pART1-pto1 transformant cells to osmotic stress also led to a rapid increase in Spc1 tyrosine phosphorylation. Thus, high expression of pto1 had no effect on the initial large increase in Spc1 tyrosine phosphorylation. However, whereas the increase in Spc1 tyrosine phosphorylation was quite transient in the control culture, decreasing to the basal amount within 50 min following exposure to osmotic stress, the level of Spc1 tyrosine phosphorylation remained high in the pART1-pto1 transformant cells for the duration of the experiment (Fig. 4). These findings directly contradict the prediction of the model, which postulates that PP2C negatively regulates Wis1 or other upstream elements of the Spc1 stress response pathway.

PP2C Does Not Regulate Spc1-dependent Phosphorylation of Atf1—Having found that Ptcl overproduction does not inhibit Spc1 tyrosine phosphorylation, we next turned our attention to the possibility that Spc1 or Atf1 is a substrate of Ptcl phosphatase. In theory, Ptcl could inhibit Spc1 by dephosphorylating the substrates of Spc1. However, whereas the increase in Spc1 tyrosine phosphorylation was quite transient in the control culture, decreasing to the basal amount within 50 min following exposure to osmotic stress, the level of Spc1 tyrosine phosphorylation remained high in the pART1-pto1 transformant cells for the duration of the experiment (Fig. 4). These findings directly contradict the prediction of the model, which postulates that PP2C negatively regulates Wis1 or other upstream elements of the Spc1 stress response pathway.

FIG. 3. Effects of the overexpression of Ptcl and Pyp phosphatases. A, high expression of the pto1 gene in pART1-pto1 transformants results in a large increase in PP2C activity. PP2C assays of Mono Q chromatographic fractionation samples from extracts of cells transformed with pART1-pto1 or a control plasmid (pART1) were performed. The assays were performed by measuring the release of radioactive phosphate from 32P-labeled casein (see “ Experimental Procedures”). B, overexpression of Ptcl or Pyp phosphatases cause different cell phenotypes. Wild type (PR109), Δwis1 (JM544), spc1-M13 (KS11363), and cells overexpressing pto1 were plated on EMM2 medium and grown 48 h at 30 °C. For pyp1 and pyp2 overexpression, PR109 cells were transformed with plasmids expressing those genes under the control of the S. pombe nmt promoter (plasmids pREP1-pyp1 and pREP1-pyp2). Transformed cells were grown on EMM2 agar medium containing thiamine, which repressed the nmt promoter. Expression was induced by plating cells on EMM2 agar lacking thiamine for 48 h at 30 °C. Bar, 20 μm.

Stress-induced Tyrosine Phosphorylation of Spc1 Is Sustained in Ptcl Overproducer Cells—To further explore the possibility that Ptcl negatively regulates Wis1 or another upstream element, we examined Spc1 tyrosine phosphorylation in

FIG. 4. Overproduction of pto1 causes tyrosine phosphorylation of Spc1 to be sustained at a high level following osmotic stress. Cells containing a chromosomal copy of Ha6H epitope-tagged spc1 and transformed with the control plasmid pART1 (control) or pART1-pto1 (ov. pto1) were incubated for the indicated times in the presence of 0.6 M KCl. Spc1 protein was precipitated with Ni2+-NTA-agarose beads and probed by Western blotting for the presence of phosphotyrosine (α-pTyr). Immunoblot analysis with an anti-HA antibody confirmed that the Spc1 levels were approximately constant throughout the experiment.
KCl  -  -  -  
Atf1
1  2  3  4
WT  ov. ptc1

FIG. 5. Phosphorylation-induced electrophoretic mobility shift of Atf1 is unaffected by overproduction of Ptc1. Cells carrying a chromosomally integrated copy of Hsap epitope-tagged atf1'' (KS1479) and transformed with the control pART1 plasmid (lanes 1 and 2; WT) or with pART1- ptc1'' plasmid (lanes 3 and 4; ov. ptc1) were grown in YES medium at 30 °C, and aliquots were harvested before (lanes 1 and 3) or after (lanes 2 and 4) 15 min of osmotic stress achieved by adjusting the culture medium to a concentration of 0.6 M KCl. Atf1-tagged protein was then purified on Ni²⁺-NTA beads and analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with anti-HA antibody. The retarded migration of Atf1 observed in the control sample (lane 2) was also seen in the sample made from cells overexpressing ptc1'' (lane 4).

dephosphorylate the sites on Atf1 that are phosphorylated by Spc1 kinase.

Transcriptional Induction of Stress Response Genes Is Reduced in Cells That Overexpress ptc1''—Although our findings strongly suggested that Ptc1 does not directly regulate Spc1 or dephosphorylate residues of Atf1 that are phosphorylated by Spc1 kinase, these studies did not directly address the possibility that Ptc1 negatively regulates the Atf1-dependent transcriptional induction of stress response genes. Therefore, studies were carried out to measure the transcriptional induction of pyp2'' and gpd1'' in pART1- ptc1'' transformant cells exposed to osmotic stress. As shown in Fig. 6, incubation of cells harboring the control plasmid pART1 in medium containing 0.6 M KCl led to the rapid and transient increase in the level of both pyp2'' and gpd1'' mRNA. These findings are consistent with previous studies (4, 10). In cells transformed with pART1- ptc1'', the pattern of pyp2'' and gpd1'' mRNA transcriptional induction was broadly similar, although the maximum level of induction was reduced by approximately 50%. These findings support the notion that Ptc1 negatively regulates the Spc1-dependent transcriptional induction of stress response genes.

DISCUSSION

Genetic Studies Indicate That PP2C Is Involved in the Negative Regulation of Stress Sensor Pathways—The major aim of the studies described herein was to test the hypothesis that type 2C protein phosphatases have as one of their major in vivo activities the negative regulation of stress-activated protein kinase cascades. Before discussing the molecular evidence presented in this paper, it may be helpful to review the genetic data that led to the formation of the model. The hypothesis arose from independent genetic studies carried out with two organisms: the budding yeast S. cerevisiae and the distantly related fission yeast S. pombe. The key findings in regard to S. cerevisiae are as follows: 1) overproduction of two PP2C genes, PTC1 and PTC3, rescue lethality induced by hyperactivation of Hog1p, a stress-activated protein kinase most similar to S. pombe Spc1 kinase; and 2) ptc1 mutations exhibit synthetic lethal interactions with mutations of PTP2, which encodes a tyrosine phosphatase (PTP) implicated in the negative regulation of Hog1p (14). These findings led Saito and colleagues (13) to propose that Ptc1p and Ptc3p negatively regulate Hog1p or Pbs2p, the latter protein being the kinase that phosphorylates and thereby activates Hog1p kinase.

The initial indication that PP2C enzymes might also be involved in the negative regulation of stress-activated kinases in fission yeast arose from the discovery via a mutant screen that the Ca²⁺-sensitive phenotype observed in a Δptc1 Δptc3 strain is suppressed by spc1 and wis1 loss-of-function mutations (16). One explanation for this observation was that loss of PP2C activity led to hyperactivation of Spc1 kinase and consequent lethality. In an attempt to extend the genetic data that relate to the model, we began our studies by investigating interactions involving PP2C and PTP genes. Genetic and biochemical studies have proven that Pyp1 and Pyp2 negatively regulate Spc1 via direct dephosphorylation of Tyr-173 (4, 10). If PP2C enzymes also negatively regulate the Spc1 kinase cascade, then one might expect to observe genetic interactions involving mutations of the PTP and PP2C genes. Indeed, we found that whereas double mutant combinations of the Δpyp1, Δptc1, and Δptc3 mutations produced cells that grew very well on standard rich growth medium, the Δpyp1 Δptc1 Δptc3 triple mutant grew very poorly. This finding is consistent with the studies of S. cerevisiae showing synthetic lethal interactions with ptc1 and ptp2 mutations (14). This correlation strongly suggests that PP2C enzymes have similar roles in regulating the stress responses in the two evolutionarily divergent yeasts. Our studies went a step further by showing that the Δpyp1 Δptc1 Δptc3 phenotype was quite effectively suppressed by Δwis1 mutations. This finding is reminiscent of the suppression of Δpyp1 Δpyp2 synthetic lethality by spc1 and wis1 mutations (4, 5). These observations are most easily interpreted to
suggest that Ptc1 and Ptc3 phosphatases are involved in the negative regulation of the Spc1 signal transduction system, although other more complex explanations cannot be excluded.

Expression of ptc1+ Is Regulated by the Stress-regulated Signal Transduction System—With a background of genetic data suggesting that PP2C enzymes negatively regulate the Spc1 kinase cascade, we proceeded to explore whether any of the known PP2C genes in S. pombe were transcriptionally induced in response to stress by an Spc1-dependent mechanism. Expression of pyp2+ is strongly elevated in cells exposed to stress, and this induction of pyp2+ mRNA transcription is entirely dependent on Spc1 and Atf1. In analogous studies, we found that the level of ptc1+ mRNA underwent a large increase in response to osmotic stress. Moreover, this response was abolished in spc1 and atf1 mutants. The similarity of findings involving the transcriptional regulation of pyp2+ and ptc1+ further supports the idea that Ptc1 may be involved in the negative regulation of the Spc1 kinase signal transduction system. As is the case for pyp2+, we propose that transcriptional induction of ptc1+ is part of a negative feedback or attenuation mechanism that serves to fine tune the cellular response to stress.

Wis1 and Spc1 Are Not Regulated by Ptc1—Tyrosine phosphorylation of Spc1 is absolutely dependent on Wis1 in vivo (4, 5). Wis1 is a MAPKK homolog, and therefore like all MAPKK homologs it is very likely that Wis1 is activated by serine and/or threonine phosphorylation carried out by a MAPKKK homolog. Indeed, recent studies have identified a MAPKKK homolog, Wik1, which activates Wik1 in response to osmotic stress (6). Wis1 can be considered to be a reasonable candidate target of Ptc1 phosphatase. If Wis1 is negatively regulated by Ptc1, then it is predicted that overproduction of Ptc1 should depress Spc1 tyrosine phosphorylation and thereby cause cells to divide at a larger size. However, we carried out such a study and found that Ptc1 overproduction did not decrease Spc1 tyrosine phosphorylation. In fact, the major effect of Ptc1 overproduction was to cause Spc1 tyrosine phosphorylation to be sustained at a high level in cells exposed to osmotic stress. Likewise, the cells that overproduced Ptc1 underwent division at a reduced cell size relative to wild type. These findings contradict a model in which Ptc1 negatively regulates Wis1. In addition, these findings are inconsistent with Spc1 inhibiting the activity of any protein that acts upstream of Wis1, such as Wik1, to promote the activation of Spc1.

Spc1 is activated by phosphorylation of two residues: Thr-171 and Tyr-173 (5). PP2C enzymes could potentially dephosphorylate Thr-171; therefore, it was reasonable to propose that Spc1 might be a direct substrate of Ptc1. Atf1 undergoes an electrophoretic mobility shift when phosphorylated by Spc1, thereby providing a convenient assay of the activation state of Spc1. The temperature sensitivity of these mutants can be measured by the MAP kinase Mpk1 implicated in cell wall metabolism (29). The temperature sensitivity of these mutants can be

FIG. 7. Model of the Spc1 cascade regulation. Upon various forms of stress, the Wik1-Wis1-Spc1 kinase cascade is stimulated, leading to the activation of the transcription factor Atf1. Active Atf1 promotes the transcription of several important genes involved in stress response, such as gpd1+. The inactivation of Spc1 is carried out by two tyrosine phosphatases: Ppy1, which is constitutively expressed; and Ppy2, whose stress-induced expression causes a negative feedback loop. The type 2C serine/threonine phosphatase encoded by ptc1+ is also transcribed in an Spc1-Atf1-dependent manner, and Ptc1 negatively regulates the pathway. Ptc1 acts downstream of Spc1, perhaps by dephosphorylating a cofactor of the Atf1-Pcr1 transcription complex (X).

Atf1 and Ptc1 co-precipitation, but these findings do not exclude a more transient or unstable interaction involving Atf1 and Ptc1.

Regulation of Stress-induced Gene Expression by Ptc1—Although our findings argue against a model in which Ptc1 dephosphorylates Spc1 or Wis1 or counteracts the phosphorylation of Atf1 that is catalyzed by Spc1, some of our molecular studies provide support for the possibility that Ptc1 regulates some aspect of the Spc1 signal transduction pathway. One important finding is that high expression of Ptc1 causes a decrease in the transcriptional induction of the pyp2+ and gpd1+ genes. This observation suggests that Ptc1 may indirectly regulate Atf1, perhaps by dephosphorylating an Atf1 co-factor involved in transcriptional induction. It was recently shown that Atf1 forms heterodimers with the protein encoded by the pcr1+ gene (28). Δpcr1 cells display a phenotype similar to Δatf1 mutant cells, suggesting that Atf1-Pcr1 heterodimers may have an important role in the stress-induced gene transcription. However, current evidence suggests that Pcr1 is not phosphorylated in vivo, suggesting that it cannot be a substrate of Ptc1 phosphatase. Nevertheless, it is likely that Atf1-Pcr1 heterodimers interact with other proteins to regulate gene expression; thus, it is plausible that these unknown factors may be substrates of PP2C enzymes as indicated in the model (Fig. 7).

The action of PP2C on this unknown cofactor could be indirect and involve another stress-activated kinase cascade. In S. cerevisiae, PTC1 was identified as a suppressor of mutation in the PKC1 gene, which encodes a protein kinase C that regulates the MAP kinase Mpk1p implicated in cell wall metabolism (29). The temperature sensitivity of these mutants can be
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