

Regulation of the *Saccharomyces cerevisiae* HOG1 Mitogen-Activated Protein Kinase by the PTP2 and PTP3 Protein Tyrosine Phosphatases

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In response to increases in extracellular osmolarity, *Saccharomyces cerevisiae* activates the HOG1 mitogen-activated protein kinase (MAPK) cascade, which is composed of a pair of redundant MAPK kinase kinases, namely, Ssk2p and Ssk22p, the MAPK kinase Pbs2p, and the MAPK Hog1p. Hog1p is activated by Pbs2p through phosphorylation of specific threonine and tyrosine residues. Activated Hog1p is essential for survival of yeast cells at high osmolarity. However, expression of constitutively active mutant kinases, such as those encoded by *SSK2ΔN* and *PBS2^{DD}*, is toxic and results in a lethal level of Hog1p activation. Overexpression of the protein tyrosine phosphatase Ptp2p suppresses the lethality of these mutations by dephosphorylating Hog1p. A catalytically inactive Cys-to-Ser Ptp2p mutant (Ptp2^{C/S}p) is tightly bound to tyrosine-phosphorylated Hog1p in vivo. Disruption of *PTP2* leads to elevated levels of tyrosine-phosphorylated Hog1p following exposure of cells to high osmolarity. Disruption of both *PTP2* and another protein tyrosine phosphatase gene, *PTP3*, results in constitutive Hog1p tyrosine phosphorylation even in the absence of increased osmolarity. Thus, Ptp2p and Ptp3p are the major phosphatases responsible for the tyrosine dephosphorylation of Hog1p. When catalytically inactive Hog1^{K/N}p is expressed in *hog1Δ* cells, it is constitutively tyrosine phosphorylated. In contrast, Hog1^{K/N}p, expressed together with wild-type Hog1p, is tyrosine phosphorylated only when cells are exposed to high osmolarity. Thus, the kinase activity of Hog1p is required for its own tyrosine dephosphorylation. Northern blot analyses suggest that Hog1p regulates Ptp2p and/or Ptp3p activity at the posttranscriptional level.

The mitogen-activated protein kinase (MAPK) cascade is a universal signal transduction network widely found in both higher and lower eukaryotic cells (19, 44). Cells have multiple MAPK cascades that are differentially triggered in response to external stimuli such as growth factors, UV radiation, and osmotic shock. A typical MAPK cascade is composed of three tiers of protein kinases: a MAPK is activated by a specific MAPK kinase (MAPKK) through phosphorylation of conserved threonine and tyrosine residues in MAPK. Phosphorylation at both residues is required for MAPK activity (5, 31). A MAPKK is activated by a specific MAPKK kinase (MAPKKK) through phosphorylation of conserved threonine and/or serine residues in MAPKK (3).

Saccharomyces cerevisiae responds to increases in osmolarity in the extracellular environment by activating a MAPK cascade, referred to as the HOG1 (high-osmolarity glycerol response) MAPK cascade (Fig. 1). This osmosensing MAPK cascade is composed of a pair of redundant MAPKKs, namely, Ssk2p and Ssk22p, the MAPKK Pbs2p, and the MAPK Hog1p (6, 7, 24, 26).

The HOG1 MAPK cascade is regulated by two independent osmosensors. One of the osmosensors is homologous to prokaryotic two-component signal transducers and is composed of three proteins (Sln1p, Ypd1p, and Ssk1p), whereas the second

osmosensor, Sho1p, is a transmembrane protein with a cytoplasmic SH3 domain (24, 26, 30, 32). Under normal (relatively low) osmotic conditions, the transmembrane histidine kinase Sln1p is catalytically active and transfers a phosphate by a phosphorelay mechanism, via Ypd1p, to the response regulator protein Ssk1p (32). Phosphorylated Ssk1p appears to be the inactive form of Ssk1p (24). In the presence of increased osmolarity, the Sln1p histidine kinase is inactivated and unphosphorylated Ssk1p is available to activate the Ssk2p and Ssk22p MAPKKs, which then activate Pbs2p and, indirectly, Hog1p. In contrast, Sho1p interacts with and activates Pbs2p, thereby bypassing the need for the Ssk2p and Ssk22p MAPKKs (24).

Activation of Hog1p, through phosphorylation of the Thr174 and Tyr176 residues, leads to transcriptional activation of several genes, including *GPD1* (NAD⁺-dependent *sn*-glycerol 3-phosphate dehydrogenase), *CTTI* (catalase T), and *HSP12* (small heat shock protein) (20, 36, 42). Increased glycerol-3-phosphate dehydrogenase activity is required to raise the intracellular glycerol concentration, a response critical to high-osmolarity adaptation (1).

The level of threonine and tyrosine phosphorylation on MAPK determines the overall level of signal output and is believed to be regulated by the opposing activities of MAPKK- and MAPK-specific protein phosphatases. In mammalian cells, dual-specificity phosphatases, which hydrolyze both phosphoserine/threonine and phosphotyrosine, appear to play an important role in the dephosphorylation of MAPK. The murine MAPK phosphatase MKP-1 (also known as 3CH134) and the human homolog CL100 dephosphorylate both phosphothreonine and phosphotyrosine residues in the MAPKs ERK1 and ERK2, as does HVH2, a human phosphatase with homology to

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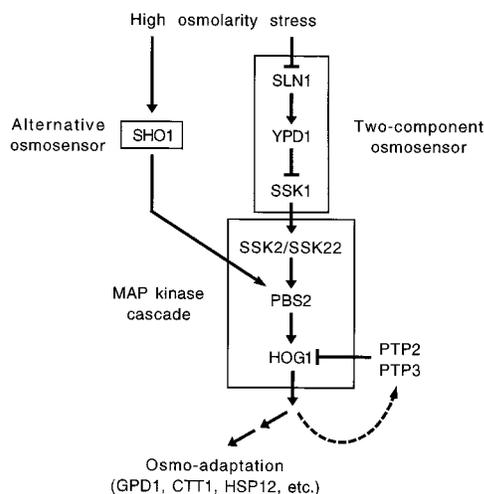


FIG. 1. The yeast HOG1 signal transduction pathway contains two independent osmosensors. The first is a two-component signal transducer and is actually composed of three proteins (Sln1p, Ypd1p, and Ssk1p), whereas the second osmosensor, Sho1p, is a transmembrane protein with a cytoplasmic SH3 domain. Under normal osmotic conditions, the transmembrane histidine kinase Sln1p is catalytically active and transfers a phosphate by a phosphorelay mechanism, via Ypd1p, to the response regulator protein Ssk1p. Phosphorylation of Ssk1p appears to inhibit the ability of Ssk1p to activate the Ssk2p and Ssk22p MAPKKKs. In the presence of increased osmolarity, the Sln1p histidine kinase is inactivated and unphosphorylated Ssk1p activates the Ssk2p and Ssk22p MAPKKKs, which in turn activate Pbs2p. Alternatively, in the presence of a high-osmolarity stimulus, Sho1p interacts with and activates Pbs2p. The activated Pbs2p then phosphorylates and activates Hog1p. Activation of Hog1p leads to the induction of transcription of genes required for adaptation to high-osmolarity stress, including *GPD1*, *CTT1*, and *HSP12*.

CL100 (4, 9, 17, 23, 41). Similarly, human PAC1, a mitogen-induced dual-specificity phosphatase, regulates the phosphorylation level of ERK2 in T lymphocytes (34, 43). In some cell types, however, ERK1 is inactivated by a combination of the serine/threonine phosphatase PP2A and a tyrosine phosphatase distinct from CL100 (2).

Although four MAPK pathways have been defined in yeast (19), very little is known about the protein phosphatases that regulate these pathways. In *S. cerevisiae*, the dual-specificity phosphatase Msg5p may play a role in the mating-pheromone-induced MAPK pathway: overexpression of *MSG5* partially inhibits *STE12*-dependent transcription, and disruption of *MSG5* leads to a slight pheromone hypersensitivity (14). In the fission yeast *Schizosaccharomyces pombe*, two tyrosine-specific phosphatases, Pyp1p and Pyp2p, act on the Spc1p MAPK (13, 37).

A clue to the identity of the protein phosphatases which act on the HOG1 MAPK pathway comes from the study of conditional lethal mutants in this pathway. Disruption of either *SLN1* or *YPD1* is lethal to yeast because this disruption leads to constitutive activation of the HOG1 MAPK cascade. Both *sln1Δ* and *ypd1Δ* mutants are rescued by overexpression of the yeast protein tyrosine phosphatase (PTPase) Ptp2p, but not by the PTPase Ptp1p (25, 26). Ptp1p and Ptp2p are cytosolic PTPases with significant sequence similarity to the large family of mammalian PTPases (16, 18, 21, 29). In this study, we examine the role of Ptp2p in the HOG1 MAPK pathway and demonstrate that Ptp2p and another yeast PTPase, Ptp3p, are the major phosphatases responsible for dephosphorylation of the tyrosine residue in Hog1p. Evidence which suggests that Hog1p regulates the activity of these phosphatases is also presented.

MATERIALS AND METHODS

Yeast strains, plasmids, media, and general methods. The *S. cerevisiae* strains and plasmids used are listed in Table 1. Growth media and standard yeast manipulations were as described elsewhere (35). *Escherichia coli* DH5 was used for all plasmid manipulations.

Buffers. Lysis buffer contains 25 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 0.1% Triton-X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamide, 5 μg of leupeptin per ml, 5 μg of pepstatin per ml, and 5 μg of chymostatin per ml. Sodium dodecyl sulfate (SDS) loading buffer contains 50 mM Tris-HCl (pH 6.8), 143 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. Stripping buffer contains 62.5 mM Tris-HCl (pH 6.7), 100 mM 2-mercaptoethanol, and 2% SDS.

Construction of chromosomal gene disruptants. *PTP3*, *MSG5*, and *YIL113* were disrupted with *HIS3* by the micro-homology-mediated PCR targeting method (27). *HOG1* was disrupted by transformation of yeast cells with an appropriate plasmid DNA fragment containing the disrupted gene. Disruption of most genes was verified by PCR analysis of chromosomal DNA, using specific primers.

Yeast cell extracts. Cells were grown in synthetic complete (SC) or YEP medium with required sugars as outlined in the figure legends. At an optical density at 600 nm of 0.5 to 0.8, approximately 10-ml cultures were harvested, resuspended in 100 μl of cold lysis buffer containing 10% glycerol, and mixed with an equal volume of chilled glass beads. After vigorous vortexing three times with 30-s pulses, samples were centrifuged at 13,000 rpm for 15 min in a micro-centrifuge at 4°C. The supernatants were removed, frozen on dry ice-ethanol, and stored at -80°C. The protein concentrations were determined by using a Bio-Rad protein assay kit, with bovine gamma globulin as the standard.

Immunoblot analysis. Samples were suspended in SDS loading buffer, immediately boiled for 4 min, and resolved by SDS-7.5 or 10% polyacrylamide gel electrophoresis. Tyrosine-phosphorylated Hog1p was detected with antiphosphotyrosine antibody 4G10 (a gift of T. Roberts). Hemagglutinin (HA) epitope-tagged Hog1p (HA-Hog1p) was detected with anti-HA monoclonal antibody 12CA5 (Boehringer Mannheim), and glutathione *S*-transferase (GST)-Ptp2p was detected with goat anti-GST (Pharmacia Biotech). Immunoblots were developed by using horseradish peroxidase-conjugated protein A/G (Pierce) and an Amersham ECL kit. Probes were removed from membranes by incubation at 65°C for 30 min in stripping buffer prior to incubation of the same membrane with another antibody.

Coimmunoprecipitation analysis. Freshly thawed cell extracts (500 μg) were incubated with 2 μg of antibody 12CA5 and 60 μl of GammaBind Plus Sepharose (50% slurry; Pharmacia Biotech) overnight while rotating at 4°C. The precipitates were washed four times with cold lysis buffer containing 150 mM NaCl and once with lysis buffer without salt. The precipitates were resuspended in 60 μl of 2× SDS loading buffer and boiled for 5 min. Immunoblot analysis was performed first with antibody 12CA5. The membrane was then stripped, reprobed with goat anti-GST antibody, stripped again, and reprobed with antiphosphotyrosine monoclonal antibody 4G10.

Northern blot analysis. Preparation of total RNA and Northern blot analyses were carried out as described previously (8). A 1.1-kb *XmnI* *PTP2* genomic DNA fragment encoding the noncatalytic region of Ptp2p was used as a probe to detect *PTP2* mRNA. A 2.3-kb *HindIII*-*BglII* *PTP3* genomic DNA fragment was used to detect *PTP3* mRNA, and a 1-kb *AvaII*-*AccI* fragment from *ACT1* was used to detect *ACT1* mRNA. Hybridizations were carried out at 42°C in the presence of 50% formamide. Probes were labeled for 60 min at 37°C with [α -³²P]dCTP, using a random-primed DNA labeling kit from Boehringer Mannheim.

RESULTS

The HOG1 MAPK pathway can be constitutively activated by either the *PBS2^{DD}* or *SSK2ΔN* mutation. The PTPase Ptp2p has been proposed to act as a negative regulator in the HOG1 pathway, since overexpression of *PTP2* suppresses the lethality of *sln1Δ* and *ypd1Δ* mutations (26). However, the step at which Ptp2p acted in this pathway was not known. To address this question, we conducted an epistasis test in which the ability of *PTP2* overexpression to suppress the lethal effects of dominant mutations in the HOG1 pathway was examined. To activate the HOG1 MAPK pathway without the involvement of upstream elements (Sln1p, Ypd1p, Ssk1p, and Sho1p), two constitutively active kinase mutations, one in *SSK2* and the second in *PBS2*, were generated. *SSK2ΔN* is a deletion mutation in which the amino-terminal inhibitory domain of the *SSK2* kinase has been removed (24). Conditional expression of *SSK2ΔN* from the *GAL1* promoter results in elevated levels of tyrosine-phosphorylated Hog1p (reference 24 and Fig. 2A). *SSK2ΔN* expression is lethal (Fig. 3), and this toxicity is re-

TABLE 1. Yeast strains and plasmids used

Strain or plasmid	Genotype ^a	Reference or source
Strains		
TM100	<i>MATa ura3 leu2 trp1</i>	26
TM101	<i>MATa ura3 leu2 his3</i>	24
TM158	<i>MATa ura3 leu2 his3 ptp2::hisG</i>	This study
TM232	<i>MATa ura3 leu2 his3 hog1::LEU2</i>	This study
SW111	<i>MATa ura3 leu2 his3 ptp2::hisG hog1::LEU2</i>	This study
SW131	<i>MATa ura3 leu2 trp1 hog1::TRP1</i>	This study
SW137	<i>MATa ura3 leu2 his3 ptp3::HIS3</i>	This study
SW139	<i>MATa ura3 leu2 his3 ptp2::hisG ptp3::HIS3</i>	This study
Plasmids		
pYES2	<i>URA3 2μm P_{GAL1}</i>	Invitrogen (San Diego, Calif.)
pGPBD21	<i>URA3 2μm P_{GAL1}-PBS2^{DD}</i> (<i>PBS2</i> with Ser514-Asp and Thr518-Asp mutations)	This study
pGSS21	<i>URA3 2μm P_{GAL1}-SSK2ΔN</i> (contains <i>SSK2</i> from Met1173 to Asp1579)	24
pDBL2	<i>LEU2 2μm P_{ADHI}</i>	28
pDBT2	<i>LEU2 2μm P_{ADHI}-PTP2</i>	26
pDBT2S	<i>LEU2 2μm P_{ADHI}-PTP2^{CIS}</i> (<i>PTP2</i> with Cys666-Ser mutation)	This study
p426-GAG3	<i>URA3 2μm P_{GAL1}-GST</i>	M. Takekawa
pSWM36	<i>URA3 2μm P_{GAL1}-GST-PTP2</i> (contains entire <i>PTP2</i> coding sequence)	This study
pSWM37	<i>URA3 2μm P_{GAL1}-GST-PTP2^{CIS}</i>	This study
pRS423	<i>HIS3 2μm</i>	39
pSWM29	<i>HIS3 2μm HA-HOG1</i> (HA epitope linked to Phe7 of Hog1p)	This study
pSSP25	<i>URA3 ARS1 CEN4 P_{GAL1}-PTP2</i>	25
pRS416	<i>URA3 CEN6 ARSH4</i>	39
pGALP2	<i>URA3 CEN6 ARSH4 P_{GAL1}</i>	This study
pSWM24	<i>URA3 CEN6 ARSH4 P_{GAL1}-HOG1</i>	This study
pHG12	<i>URA3 CEN6 ARSH4 HOG1</i>	This study
pSWM15	<i>URA3 CEN6 ARSH4 HOG1^{KIN}</i> (<i>HOG1</i> with Lys52-Asn mutation)	This study
pRS426- <i>PTP3</i>	<i>URA3 2μm PTP3</i>	K. L. Guan
pRS306- <i>P_{GAL1}-ACT1</i>	<i>URA3 CEN6 P_{GAL1}-ACT1</i>	R. Li

^a *P_{GAL1}* and *P_{ADHI}* are, respectively, *GAL1* and *ADHI* promoters.

versed by disruption of either of the two downstream genes, *PBS2* or *HOG1* (24). The *PBS2^{DD}* allele contains two mutations that replace both of the phosphorylation sites required for Pbs2p activation, namely, Ser514 and Thr518, with Asp. These substitution mutations mimic the activating phosphorylation events (3) and activate Pbs2p without the involvement of upstream elements (Ssk2p, Ssk22p, and Sho1p). Expression of *PBS2^{DD}* from the *GAL1* promoter results in constitutively elevated levels of tyrosine-phosphorylated Hog1p (Fig. 2A) and is lethal to yeast cells (Fig. 3). This *PBS2^{DD}* lethality is dependent on the presence of *HOG1* (data not shown).

Overexpression of either wild-type or catalytically inactive Cys666-Ser mutant *PTP2* suppresses the lethality caused by expression of *PBS2^{DD}* or *SSK2ΔN*. The ability of *PTP2* overexpression to suppress the lethality generated by the *SSK2ΔN* and *PBS2^{DD}* mutations was then investigated. Expression of *SSK2ΔN* or *PBS2^{DD}* from the *GAL1* promoter (*P_{GAL1}*) was not lethal in cells that constitutively expressed *PTP2* from the *ADHI* promoter (*P_{ADHI}*) (Fig. 3). The ability of Ptp2p to suppress the lethality of the *SSK2ΔN* and *PBS2^{DD}* mutations indicates that Ptp2p acts at the level of or downstream of the Ptp2p MAPKK. However, since Pbs2p is not tyrosine phosphorylated, it seemed more likely that Ptp2p was acting after Pbs2p, most likely at the level of Hog1p. We then examined if suppression by *PTP2* overexpression is dependent on Ptp2p phosphatase activity. For this purpose, we generated a catalytically inactive mutant enzyme (Ptp2^{CIS}p) which contains a serine mutation at cysteine residue 666 (Cys666-Ser) in the phosphatase domain. Cys666 corresponds to the catalytic nucleophile of all PTPases, and enzymes containing a mutation at this site are catalytically inactive (10, 33, 40). Surprisingly, constitutive expression of *ptp2^{CIS}* also suppressed the lethality generated by expression of the *SSK2ΔN* and *PBS2^{DD}* muta-

tions (Fig. 3). Thus, it appeared that the Ptp2p phosphatase activity was not essential to the suppression process. However, further studies demonstrated that the suppression mechanisms for Ptp2p and Ptp2^{CIS}p are different.

To gain insight into how the catalytically inactive Ptp2^{CIS}p suppresses the *PBS2^{DD}* lethality, the level of tyrosine-phosphorylated Hog1p was examined in cells expressing *PBS2^{DD}* and either *PTP2⁺* or *ptp2^{CIS}*. In control cells containing pGPBD21 (*P_{GAL1}-PBS2^{DD}*) and pDBL2 (*P_{ADHI}* vector), elevated levels of tyrosine-phosphorylated Hog1p were observed 30 min after induction of *PBS2^{DD}* (Fig. 2B, lanes 1 to 4). In the presence of elevated levels of *PTP2⁺*, however, almost no tyrosine-phosphorylated Hog1p was observed (Fig. 2B, lanes 5 to 8). In contrast, expression of *ptp2^{CIS}*, which also suppresses the lethality of the *PBS2^{DD}* mutation, resulted in elevated levels of tyrosine-phosphorylated Hog1p, even in the absence of *PBS2^{DD}* expression (Fig. 2B, lanes 9 to 12).

Overexpression of *PTP2⁺* or *ptp2^{CIS}* also resulted in different levels of tyrosine-phosphorylated Hog1p in wild-type cells in which the *HOG1* pathway was activated by an increase in extracellular osmolarity (rather than through *PBS2^{DD}* expression). Overexpression of *PTP2⁺* almost completely abrogated the Hog1p tyrosine phosphorylation observed when control cells were exposed to a high osmolarity (Fig. 2C). In contrast, overexpression of *ptp2^{CIS}* results in elevated levels of tyrosine-phosphorylated Hog1p even in the absence of increased osmolarity. Thus, these results are consistent with the interpretation that overexpression of *PTP2⁺* suppresses the lethality of *PBS2^{DD}* or *SSK2ΔN* expression by lowering the level of tyrosine-phosphorylated Hog1p. However, the mechanism through which overexpression of *ptp2^{CIS}* suppresses the same lethality must be different.

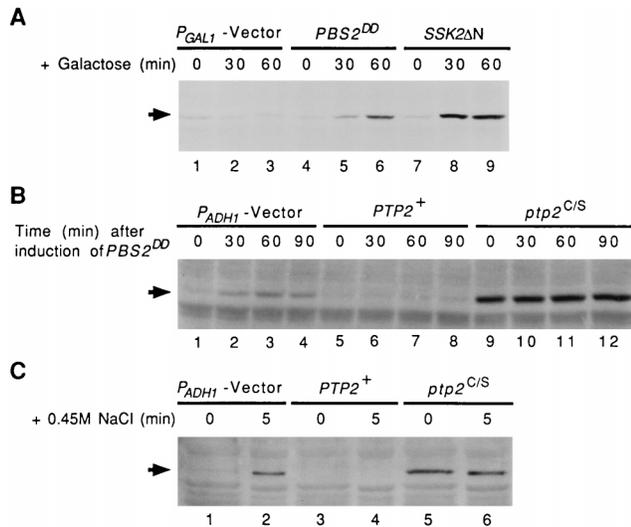


FIG. 2. Effects of the PTPase Ptp2p on Hog1p tyrosine phosphorylation were analyzed by Western blotting using antiphosphotyrosine antibody 4G10. (A) The HOG1 MAPK pathway is constitutively activated by the *PBS2^{DD}* or *SSK2ΔN* mutation. TM101 (wild-type) cells containing pYES2 (*P_{GAL1}* vector), pGPBD21 (*P_{GAL1}-PBS2^{DD}*), or pGSS21 (*P_{GAL1}-SSK2ΔN*) were grown in SC minus uracil plus 2% raffinose. Galactose was added to 2.5%, and samples were taken at the indicated time points. Each lane contains 400 μ g of cell extract. (B) Hog1p phosphotyrosine levels in cells expressing *PBS2^{DD}* that are suppressed by overexpression of either wild-type *PTP2* or a *ptp2^{C/S}* mutation. TM101 cells containing pGPB21 (*P_{GAL1}-PBS2^{DD}*) and either pDBL2 (*P_{ADH1}* vector), pDBT2 (*P_{ADH1}-PTP2⁺*), or pDBT2S (*P_{ADH1}-ptp2^{C/S}*) were grown in SC minus uracil and leucine plus 2% raffinose. Galactose was added to 2.5%, and samples were taken at the indicated time points. Each lane contains 400 μ g of extract. (C) Overexpression of *PTP2⁺* or a *ptp2^{C/S}* mutation alters the level of tyrosine-phosphorylated Hog1p following high-osmolarity stimulation. TM101 cells containing either pDBL2 (*P_{ADH1}* vector), pDBT2 (*P_{ADH1}-PTP2⁺*), or pDBT2S (*P_{ADH1}-ptp2^{C/S}*) were grown in SC minus leucine plus glucose. Cells were harvested before and after addition of NaCl to 0.45 M for 5 min. Each lane contains 150 μ g of extract.

Ptp2^{C/S}p binds to Hog1p. One possible mechanism through which expression of *ptp2^{C/S}* induces hyper-tyrosine phosphorylation of Hog1p is by binding to phosphorylated Hog1p, thereby preventing its dephosphorylation by Ptp2p or some other phosphatase. To test this possibility, GST-Ptp2p or GST-Ptp2^{C/S}p fusion protein and HA-Hog1p were coexpressed in

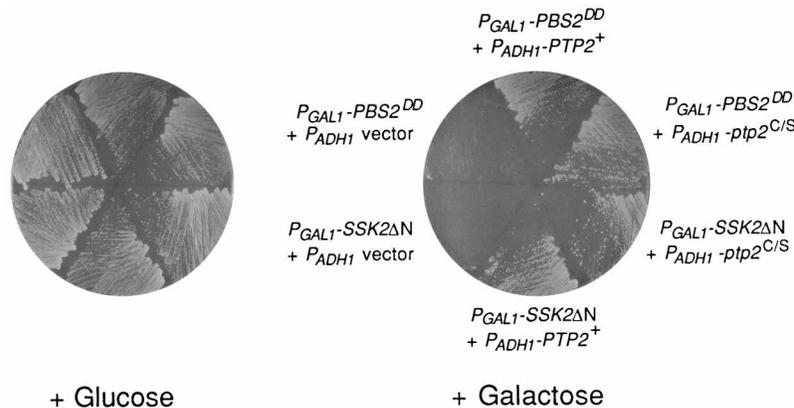


FIG. 3. Overexpression of wild-type *PTP2* or a *ptp2^{C/S}* mutation rescues cells from the lethality generated by the *PBS2^{DD}* and *SSK2ΔN* mutations. TM101 cells were transformed with either pGPB21 (*P_{GAL1}-PBS2^{DD}*) in combination with pDBL2 (*P_{ADH1}* vector), pDBT2 (*P_{ADH1}-PTP2⁺*), or pDBT2S (*P_{ADH1}-ptp2^{C/S}*) or with pGSS21 (*P_{GAL1}-SSK2ΔN*) in combination with pDBL2 (*P_{ADH1}* vector), pDBT2 (*P_{ADH1}-PTP2⁺*), or pDBT2S (*P_{ADH1}-ptp2^{C/S}*). Transformants were grown on SC minus uracil and leucine plates containing glucose or galactose and grown at 30°C.

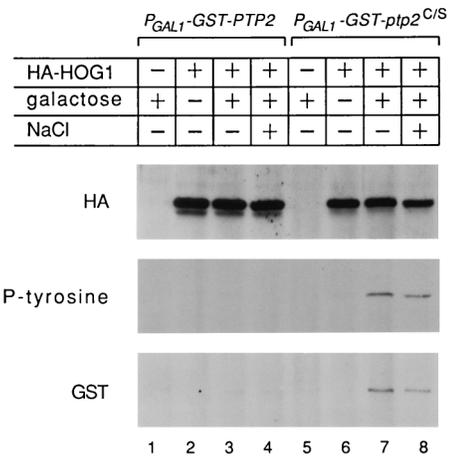


FIG. 4. Coimmunoprecipitation of HA-Hog1p and GST-Ptp2^{C/S}p. Yeast strain SW111 (*ptp2Δ hog1Δ*) containing pSWM36 (*P_{GAL1}-GST-PTP2*) or pSWM37 (*P_{GAL1}-GST-ptp2^{C/S}*) and either pRS423 (control plasmid; -) or pSWM29 (*HA-HOG1*; +) was grown in SC minus uracil and histidine plus 2% raffinose. Galactose was added to 2.5%, and cells were incubated at 30°C for 3 h. Cells were harvested before (-) and 5 min after (+) addition of NaCl to 0.45 M. HA-Hog1p was immunoprecipitated from 500 μ g of extract with anti-HA antibody 12CA5. Samples were analyzed by sequential Western blotting with the 12CA5, anti-GST, and 4G10 antibodies.

yeast cells, and coimmunoprecipitation experiments were carried out. *HA-HOG1* suppressed the osmosensitivity of a *hog1Δ* strain, indicating that it encodes a functional protein (data not shown). HA-Hog1p was precipitated from total cell extracts with the anti-HA antibody 12CA5, and the precipitates were analyzed by sequential immunoblotting using the anti-HA (12CA5), anti-GST, and antiphosphotyrosine (4G10) antibodies. GST-Ptp2^{C/S}p coprecipitates with HA-Hog1p, and the HA-Hog1p present in these precipitates is tyrosine phosphorylated in cells cultured in normal or high-osmolarity medium (Fig. 4, lanes 7 and 8). No wild-type GST-Ptp2p could be coprecipitated with HA-Hog1p (Fig. 4, lanes 3 and 4). The inability to coprecipitate wild-type GST-Ptp2p and HA-Hog1p was not due to the absence of the GST-Ptp2p protein in the extracts, since Western blot analyses performed with the anti-GST antibody on total extracts demonstrated that GST-Ptp2p and GST-Ptp2^{C/S}p were present in approximately equivalent

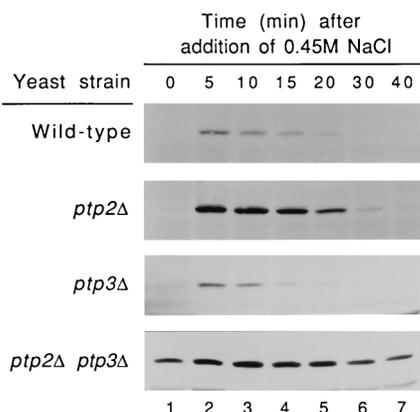


FIG. 5. The PTPases Ptp2p and Ptp3p regulate the level of tyrosine phosphorylation of Hog1p. Yeast strains TM101 (wild type), TM158 (*ptp2Δ*), SW137 (*ptp3Δ*), and SW139 (*ptp2Δ ptp3Δ*) were grown in YPD medium. NaCl was added to a final concentration of 0.45 M, and cells were harvested at the indicated time points. Four hundred micrograms of extract was analyzed by Western blotting with antiphosphotyrosine antibody 4G10.

amounts (data not shown). The HA-Hog1p precipitated from extracts prepared from cells containing wild-type GST-Ptp2p was not tyrosine phosphorylated even when the extracts were prepared from salt-treated cells. The inability to detect wild-type GST-Ptp2p in the HA-Hog1p immunoprecipitates is, therefore, most likely due to the rapid dissociation of the HA-Hog1p substrate from the Ptp2p phosphatase once HA-Hog1p is dephosphorylated. In contrast, GST-Ptp2^{C/S}p, which is unable to dephosphorylate HA-Hog1p, remains bound to the substrate.

The binding of GST-Ptp2^{C/S}p to Hog1p *in vivo* explains the ability of Ptp2^{C/S}p to suppress the lethality generated by the *SSK2ΔN* or *PBS2^{DD}* mutation even though the level of tyrosine-phosphorylated Hog1p remains high. In these mutants, binding of Ptp2^{C/S}p to activated Hog1p sequesters Hog1p, preventing not only its dephosphorylation by tyrosine phosphatases but also the ability of Hog1p to activate other proteins. While Ptp2^{C/S}p sequesters a sufficient level of activated Hog1p to suppress the lethality of *SSK2ΔN* or *PBS2^{DD}*, these cells are not osmosensitive (data not shown), indicating that at least a low level of Hog1p is available to allow the cells to properly adapt to increases in osmolarity.

***PTP2* and *PTP3* encode the major tyrosine phosphatases specific to Hog1p *in vivo*.** The preceding results demonstrate that Ptp2p can dephosphorylate the Hog1p phosphotyrosine residue and has a high affinity to tyrosine-phosphorylated Hog1p. However, because these experiments involved overexpression of the phosphatase, it was not clear if Ptp2p was a physiologically relevant Hog1p tyrosine phosphatase. Thus, we investigated the effect of the disruption of *PTP2* on Hog1p tyrosine phosphorylation *in vivo*. In wild-type cells, following exposure to increased osmolarity, the level of tyrosine-phosphorylated Hog1p reaches a peak by 5 min and then declines, returning to prestimulation levels by 20 min (Fig. 5). In the absence of *PTP2*, the overall level of tyrosine-phosphorylated Hog1p is significantly higher and the return to prestimulation levels is delayed until 30 to 40 min. In comparable experiments using an epitope-tagged Hog1p (HA-Hog1p), the level of HA-Hog1p did not change significantly during the course of the experiment (data not shown).

Although disruption of *PTP2* has a major effect on the level of Hog1p tyrosine phosphorylation, the absence of Ptp2p does

not completely prevent Hog1p tyrosine dephosphorylation. Thus, at least one other tyrosine phosphatase must act on Hog1p. Based on their amino acid sequences, several phosphatases presented themselves as possible candidates: two PTPases encoded by *PTP1* (18, 21) and *PTP3* (16a [*PTP3* is identical to the Saccharomyces Genome Database open reading frame *YER075*]), and two dual-specificity phosphatases encoded by *MSG5* and *YIL113*. Msg5p was reported to act on the Fus3p MAPK in the mating pheromone response pathway (14). The *YIL113* gene, which was identified by the yeast genome project, encodes a protein of 209 amino acids that is 56% identical to Msg5p.

PTP1, *PTP3*, *MSG5*, and *YIL113* were disrupted independently and also in combination with a *PTP2* disruption. The effects of these disruptions on Hog1p tyrosine phosphorylation were then examined by antiphosphotyrosine immunoblot analyses. Disruption of *PTP1*, *MSG5*, or *YIL113*, alone or in the presence of *ptp2Δ*, had no additional effect on Hog1p tyrosine phosphorylation (data not shown). Furthermore, an *msg5Δ yil113Δ* double mutant had the same Hog1p tyrosine phosphorylation kinetics as wild-type (*MGS5⁺ YIL113⁺*) cells, and a *ptp2Δ msg5Δ yil113Δ* triple mutant was indistinguishable from the parental *ptp2Δ* single mutant (data not shown). More important, although disruption of *PTP3* alone had no effect on the level of tyrosine phosphorylation of Hog1p (or any other protein), disruption of both *PTP2* and *PTP3* led to a significant change in the level of tyrosine-phosphorylated Hog1p (Fig. 5). In *ptp2Δ ptp3Δ* double-mutant cells, Hog1p was constitutively phosphorylated on tyrosine at a high level both in the absence and in the presence of increased osmolarity.

ptp2Δ ptp3Δ cells are viable when *HOG1* is present in single copy (i.e., chromosomal *HOG1*). However, overexpression of *HOG1* from the *P_{GAL1}*, while not lethal in *ptp2Δ* or *ptp3Δ* single-mutant cells, is lethal in *ptp2Δ ptp3Δ* double-mutant cells (Fig. 6). The ability of *ptp2Δ ptp3Δ* cells to tolerate a single copy of *HOG1* may be because only a small percentage of the constitutively tyrosine-phosphorylated Hog1p is also threonine phosphorylated. The lethality resulting from *HOG1* overexpression could then be due to an increase in the basal level of Hog1p that is threonine and tyrosine phosphorylated.

Hog1p kinase activity induces PTPase activity. It was recently demonstrated that the *S. pombe* MAPK Spc1p partially regulates its own dephosphorylation by transcriptionally activating the PTPase Pyp2p (13). However, Spc1p does not appear to regulate the level of Pyp1p, a second Spc1p-specific PTPase (13, 37). To determine if Hog1p plays a role in regulating its own dephosphorylation, we generated a catalytically inactive *HOG1* mutation, *hog1^{K/N}*, which contains the Lys52-Asn mutation in the ATP binding site. *hog1^{K/N}* is unable to suppress the osmosensitivity of *hog1Δ* strains (data not shown), indicating that Hog1^{K/N}p is an inactive MAPK. However, the tyrosine and threonine residues that are phosphorylated by Pbs2p are intact in Hog1^{K/N}p. Antiphosphotyrosine immunoblots demonstrated that Hog1^{K/N}p can indeed be tyrosine phosphorylated. However, when Hog1^{K/N}p is expressed in a *hog1Δ* strain, it is tyrosine phosphorylated even in the absence of a high-osmolarity stimulus (Fig. 7A).

Constitutive phosphorylation of Hog1^{K/N}p in a *hog1Δ* strain could be due to the inability of the catalytically inactive Hog1^{K/N}p kinase to generate the signal required to induce a Hog1p-specific tyrosine phosphatase. Alternatively, Hog1^{K/N}p may be a poor substrate for Ptp2p and Ptp3p. To distinguish between these two models, we tested whether constitutive phosphorylation of Hog1^{K/N}p occurs when wild-type Hog1p is present in the same cell. The first model predicts that the presence of wild-type Hog1p promotes the dephosphorylation

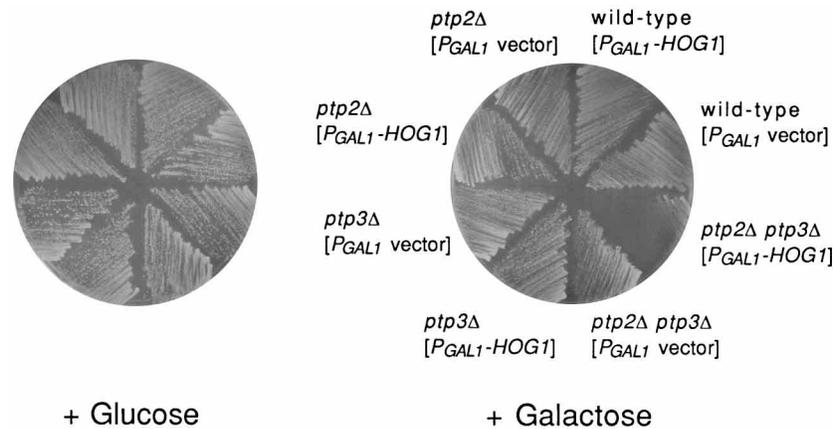


FIG. 6. Overexpression of *HOG1* is lethal in *ptp2Δ ptp3Δ* cells. Strains TM101 (wild type), TM158 (*ptp2Δ*), SW137 (*ptp3Δ*), and SW139 (*ptp2Δ ptp3Δ*) were each transformed with pGALP2 (a control plasmid containing *P_{GAL1}*) or pSWM24 (*P_{GAL1}-HOG1*). Transformants were grown onto SC minus uracil plates containing glucose or galactose and incubated at 30°C.

of Hog1^{K/N}p, while the second model predicts that Hog1^{K/N}p is constitutively phosphorylated irrespective of the presence of the wild-type Hog1p. Wild-type Hog1p or Hog1^{K/N}p (carried on a single-copy plasmid with the endogenous *HOG1* promoter) was expressed in a wild-type or *hog1Δ* strain, and the level of tyrosine phosphorylation of Hog1p (and/or Hog1^{K/N}p) in the absence and presence of increased osmolarity was examined. In the *hog1Δ* strain, Hog1^{K/N}p was tyrosine phosphorylated in the absence and presence of a high-osmolarity stim-

ulus (Fig. 7, lanes 11 and 12). However, in wild-type cells (i.e., in the presence of Hog1p), tyrosine phosphorylation of Hog1^{K/N}p was observed only following high-osmolarity stimulation (Fig. 7, lanes 5 and 6). When Hog1^{K/N}p and HA-Hog1p were coexpressed in a *hog1Δ* strain, both Hog1^{K/N}p and HA-Hog1p, which migrate at different positions in the SDS-polyacrylamide gel, were detected in antiphosphotyrosine immunoblots (data not shown). Thus, it is likely that activation of Hog1p is required to induce the phosphatase activity that removes phosphotyrosine from Hog1p. Combined with the results from the preceding sections, these findings indicate that the activity of Ptp2p and/or Ptp3p PTPases is induced by the activated Hog1p MAPK.

Transcriptional control is an important mechanism used to regulate the level of phosphatases that act on specific MAPKs, including mammalian MKP-1 and PAC1 and the *S. pombe* *pyp2*⁺ gene product (9, 13, 15). To determine if *PTP2* and *PTP3* expression in *S. cerevisiae* is controlled at the transcriptional level following high-osmolarity stimulation, Northern blot analysis was performed with RNA prepared from cells that had been exposed to 0.45 M NaCl. In contrast to *S. pombe* *pyp2*⁺, which is not expressed prior to high-osmolarity shock, both *PTP2* and *PTP3* mRNAs are present in wild-type cells prior to the addition of NaCl (Fig. 8A and B, lanes 2). Following exposure to high salt, the levels of *PTP2* and *PTP3* mRNAs increase only slightly by 20 min (Fig. 8). These small increases in *PTP* mRNA levels are unlikely to be a significant contributing factor for Hog1p tyrosine dephosphorylation, because Hog1p tyrosine phosphorylation is maximal by 5 min and returns to prestimulation levels by 15 to 20 min. Both *PTP2* and *PTP3* mRNAs are present in *hog1Δ* mutant cells, further supporting the conclusion that Hog1p activity is not required for their expression (data not shown). Furthermore, the protein synthesis inhibitor cycloheximide had no significant effect on the time course of tyrosine phosphorylation and dephosphorylation of Hog1p in wild-type cells (data not shown). These data thus suggest that Hog1p controls Ptp2p and/or Ptp3p activities at the posttranslational level.

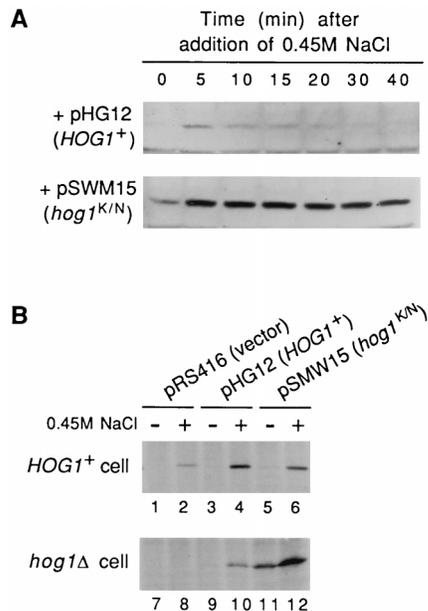


FIG. 7. (A) The Hog1^{K/N}p mutant protein is constitutively tyrosine phosphorylated. Yeast strain SW131 (*hog1Δ*) transformed with either pHG12 (*HOG1*⁺) or pSWM15 (*hog1*^{K/N}) was grown in SC minus uracil plus glucose. NaCl was added to a final concentration of 0.45 M, and samples were taken at the indicated time points. Two hundred fifty micrograms of extract was analyzed by Western blotting with antiphosphotyrosine antibody 4G10. (B) *trans*-dominant effect of wild-type Hog1p over the Hog1^{K/N}p mutant protein. Strains TM101 (wild type) and TM232 (*hog1Δ*) were transformed with pRS416 (control vector), pHG12 (*HOG1*⁺), or pSWM15 (*hog1*^{K/N}). Transformants were grown in SC minus uracil plus glucose. Cells were harvested before (–) and 5 min after (+) addition of NaCl to 0.45 M. Cell pellets were boiled in SDS sample buffer and analyzed by Western blotting with antiphosphotyrosine antibody 4G10.

DISCUSSION

To adapt to increases in extracellular osmolarity, *S. cerevisiae* activates the Hog1p MAPK through phosphorylation of threonine (Thr174) and tyrosine (Tyr176) residues. Dephos-

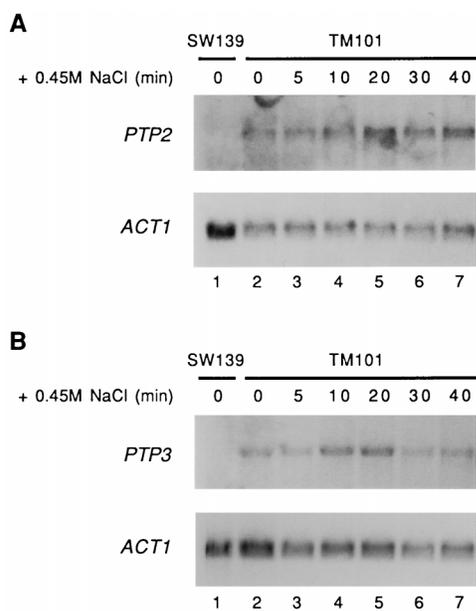


FIG. 8. Northern blot analysis of *PTP2* and *PTP3* mRNAs. TM101 (wild-type) cells were grown in YPD medium. NaCl was added to a final concentration of 0.45 M, and samples were taken at the indicated time points. SW139 (*ptp2Δ ptp3Δ*) cells were grown in YPD in the absence of added NaCl and are included as the specificity control for the *PTP2* and *PTP3* probes. Forty-five micrograms of total RNA per time point was analyzed by Northern blotting. The filters were probed sequentially for *PTP2* and *ACT1* (A) or *PTP3* and *ACT1* (B).

phorylation of these residues is essential to prevent the prolonged activation of the HOG1 MAPK cascade, which is toxic, and to reset the signaling pathway so that the cell can respond to further changes in its extracellular environment. We demonstrate here that Ptp2p and Ptp3p are the two tyrosine phosphatases primarily responsible for the dephosphorylation of the tyrosine residue in Hog1p. Disruption of *PTP2* results in significantly increased levels of tyrosine-phosphorylated Hog1p immediately following high-osmolarity stimulation and in a prolonged period before returning to the prestimulation level of unphosphorylated Hog1p. Disruption of *PTP2* together with *PTP3* results in constitutively high levels of tyrosine-phosphorylated Hog1p in the absence or presence of increased osmolarity.

Overexpression of either wild-type *PTP2* or a *ptp2^{C/S}* mutation suppresses the lethality of the *SSK2ΔN* or *PBS2^{DD}* mutation, both of which constitutively activate Hog1p. Suppression by wild-type Ptp2p occurs by inactivating Hog1p through dephosphorylation of the phosphotyrosine residue. In contrast, suppression by Ptp2^{C/S}p occurs through binding of Ptp2^{C/S}p to activated Hog1p. This binding prevents not only dephosphorylation of the Hog1p phosphotyrosine residue but also the ability of the activated Hog1p to transmit a downstream signal. The ability of Ptp2^{C/S}p to accumulate tyrosine-phosphorylated Hog1p in the absence of external stimulation is similar to the case of mammalian MAPK phosphatase MKP-1 and the ERK2 MAPK. Expression of a catalytically inactive cysteine-to-serine mutant of MKP-1 accumulates the tyrosine-phosphorylated form of ERK2 MAPK in the absence of serum stimulation (41). However, in the experiments carried out with the *ptp2^{C/S}* mutation, only the tyrosine phosphorylation level of Hog1p was examined. Because Hog1p kinase activity is also dependent on threonine phosphorylation, it is formally possible that in binding to Ptp2^{C/S}p, activated Hog1p becomes more acces-

sible to a serine/threonine phosphatase. Dephosphorylation of the threonine residue in Hog1p would result in inactivation of the kinase and contribute to the suppression of the *PBS2^{DD}* lethality. Until the level of Hog1p threonine phosphorylation in the presence of Ptp2^{C/S}p is examined, this model cannot be excluded.

In mammalian and yeast cells, MAPK inactivation can occur through the action of dual-specificity phosphatases or through the action of a serine/threonine phosphatase in combination with a tyrosine phosphatase. In mammalian cells, at least four dual-specificity phosphatases that act, with different specificities, on the three mammalian MAP kinases thus far identified have been found (4, 9, 17, 23, 41). MAPK inactivation by the combination of a serine/threonine phosphatase and a PTPase appears to depend on the cell type and may also depend on the subcellular location of the MAPK (2, 22). In *S. cerevisiae*, inactivation of the Fus3p MAPK may be regulated by a dual-specificity phosphatase, Msg5p (14), while Hog1p is inactivated most probably by a combination of tyrosine phosphatases and serine/threonine phosphatases. Although dephosphorylation of Tyr176 in Hog1p is regulated by Ptp2p and Ptp3p, the enzyme(s) responsible for dephosphorylating Thr174 is unknown. It is possible that the type 2C serine/threonine phosphatase Ptc1p acts on Hog1p, because overexpression of Ptc1p suppresses the lethality of *shn1Δ* and *ypd1Δ* mutations (references 25 and 26 and our unpublished observation). A similar type 2C phosphatase in *S. pombe*, Ptc1p, has also been implicated in the down-regulation of an osmoregulatory MAPK cascade (38). In both cases, however, it is equally possible that the target of the type 2C protein phosphatase is the MAPKK, which is activated by threonine/serine phosphorylation.

The experiments carried out with Hog1^{K/N}p demonstrate that Hog1p negatively regulates its own tyrosine dephosphorylation through control of Ptp2p and Ptp3p. Hog1^{K/N}p is an inactive kinase due to a mutation in the ATP binding site and cannot suppress the osmosensitivity of a *hog1Δ* strain. Unlike wild-type Hog1p, Hog1^{K/N}p is tyrosine phosphorylated even in the absence of a high-osmolarity stimulus. The constitutive level of Hog1^{K/N}p tyrosine phosphorylation in a *hog1Δ* strain is similar to that of wild-type Hog1p in a *ptp2Δ ptp3Δ* strain, suggesting that the kinase activity of Hog1p is required to induce Ptp2p and Ptp3p activity. This hypothesis was confirmed by expressing Hog1^{K/N}p in wild-type (*HOG1⁺*) cells. In the presence of wild-type Hog1p, Hog1^{K/N}p is tyrosine phosphorylated only in the presence of high osmolarity. The fact that Hog1^{K/N}p is tyrosine phosphorylated in *hog1Δ* cells in the absence of osmotic stress also suggests that a low basal level of signal is transmitted through the HOG1 MAPK pathway. In wild-type cells, this signal is dampened by Hog1p induction of Ptp2p and Ptp3p activity. Finally, the possibility that the level of Hog1p phosphorylation may also be affected, at least partly, by the ability of Hog1p to negatively regulate upstream signaling proteins must be noted.

At what level does Hog1p regulate Ptp2p and Ptp3p activity? Within 5 min of high-osmolarity stimulation, Hog1p is phosphorylated to maximal levels by Pbs2p. If Hog1p regulated *PTP2* and/or *PTP3* at the transcriptional level, one would have expected to see an increase in *PTP2* and/or *PTP3* mRNA levels following the peak activation of Hog1p. This was not the case, however. Under these conditions, transcription from *PTP2* and *PTP3* increased only slightly. Furthermore, both *PTP2* and *PTP3* mRNA are present in *hog1Δ* mutant cells, indicating that Hog1p activity is not required for their expression. These experiments distinguish Hog1p from its counterpart in *S. pombe*, the osmotic stress-stimulated MAPK Spc1p. Upon exposure of *S. pombe* cells to high salt, Spc1p tyrosine phosphorylation, like

that of Hog1p, increases to a maximal level within 5 min. However, in *S. pombe*, *pyp2*⁺ mRNA, which is undetectable prior to osmotic stress, increases to a very high level by 20 min. In the absence of *spc1*⁺, no *pyp2*⁺ mRNA is observed before or after high-osmolarity stress (13).

Although Hog1p could regulate Ptp2p and Ptp3p activity through translational control, a posttranslational mechanism seems more likely since the protein synthesis inhibitor cycloheximide did not affect the phosphorylation kinetics of Hog1p. Possibly, Hog1p directly phosphorylates Ptp2p and/or Ptp3p, resulting in elevated PTPase activity. Both Ptp2p and Ptp3p contain the potential MAPK phosphorylation site Pro-X-Ser-Pro (12) in their amino-terminal noncatalytic domains. The function of this amino-terminal domain is not known, but it is possible that in its unphosphorylated state, it inhibits the phosphatase domain. Alternatively, Hog1p could modify a regulatory protein that either activates or inhibits Ptp2p and/or Ptp3p. This regulatory protein may normally inhibit Ptp2p and/or Ptp3p activity unless phosphorylated by Hog1p. It is also possible that the regulatory protein is an activator of Ptp2p and/or Ptp3p that remains dormant until phosphorylated by Hog1p.

Exposure of yeast cells to hypotonic shock results in activation of another MAPK, Mpk1p, that is regulated by the PKC1 signal pathway (11). Since Hog1p and Mpk1p mediate responses to opposing extracellular osmolarity conditions, it may be advantageous to cells to transiently inactivate Mpk1p to ensure optimal adaptation to hypertonic shock. An interesting possibility is that the activation of Hog1p indirectly down-regulates Mpk1p through the activities of Ptp2p and/or Ptp3p. Thus, these protein tyrosine phosphatases may have a function in coordinating the activities of disparate MAPK signaling cascades.

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