Two Protein-tyrosine Phosphatases Inactivate the Osmotic Stress Response Pathway in Yeast by Targeting the Mitogen-activated Protein Kinase, Hog1*

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Protein phosphatases inactivate mitogen-activated protein kinase (MAPK) signaling pathways by dephosphorylating components of the MAPK cascade. Two genes encoding protein-tyrosine phosphatases, PTP2, and a new phosphatase, PTP3, have been isolated in a genetic selection for negative regulators of an osmotic stress response pathway called HOG, for high osmolarity glycerol, in budding yeast. PTP2 and PTP3 were isolated as multicopy suppressors of a severe growth defect due to hyperactivation of the HOG pathway. Phosphatase activity is required for suppression since mutation of the catalytic Cys residue in Ptp2 and Ptp3, destroys suppressor function and biochemical activity. The substrate of these phosphatases is likely to be the MAPK, Hog1. Catalytically inactive Ptp2 and Ptp3 co-preficate with Hog1 from yeast extracts. In addition, strains lacking PTP2 and PTP3 do not dephosphorylate Hog1-phosphotyrosine as well as wild type. The latter suggests that PTP2 and PTP3 play a role in adaptation. Consistent with this role, osmotic stress induces expression of PTP2 and PTP3 transcripts in a Hog1-dependent manner. Thus Ptp2 and Ptp3 likely act in a negative feedback loop to inactivate Hog1.

MAPK1 signaling is ubiquitous among eukaryotes and regulates a variety of processes. In metazoans and in yeast, MAPK pathways regulate growth, development, and the response to stress (reviewed in Refs. 1–5). In Drosophila, Caenorhabditis elegans, and vertebrates, receptor tyrosine kinases, acting through Ras and Raf, activate MEK and MAPK, regulating growth and development. In vertebrates, JNK/SAPK, and p38, inhibit MAPK, Hog1. This negative regulatory pathway is thought to be a two-component system. Although mechanisms that activate MAPK pathways have been well characterized, mechanisms that inactivate these pathways are not as well understood. Protein phosphatases negatively regulate MAPK pathways, but the identity of the physiologically relevant phosphatases and their targets is unclear. MEK is inactivated in vitro by the vertebrate Ser/Thr phosphatase, PP2A (6), but it is not established that this occurs in vivo. More is known about the inactivation of MAPK. Dual specificity phosphatases in vertebrates and Saccharomyces cerevisiae inactivate MAPKs in vitro and in vivo. The vertebrate MKP-1 inactivates ERK1 and ERK2 in vitro, but it is not certain that this occurs in vivo (11–14). The yeast Msg5 inactivates Fus3 in vitro and in vivo (15). The vertebrate Ser/Thr phosphatase PP2A inactivates MAPK in vitro (16), but it has not been established that this occurs in vivo. Cells treated with okadaic acid, an inhibitor of PP2A, activate MAPK in vitro (17). Since okadaic acid also activates MEK, it is not clear that PP2A inactivates MAPK in vivo (13, 17, 18). Two protein-tyrosine phosphatases, Pyp1 and Pyp2 in Schizosaccharomyces pombe, inactivate Sty1/Spc1 in vitro and in vivo (19–21). In S. cerevisiae, a putative protein-tyrosine phosphatase encoded by PTP2 (22–24) negatively regulates the osmotic stress response pathway, and indirect evidence suggests this occurs by dephosphorylation of Hog1-phosphotyrosine (Hog1-Tyr(P)) (25).

We sought to examine further the regulation of MAPK pathways by identifying and characterizing protein phosphatases that act on the HOG pathway in S. cerevisiae. This pathway allows yeast to grow in high osmolarity environments by inducing the expression of osmoprotectants via activation of the MAPK module, Pbs2-Hog1 (Fig. 1) (26). Upstream of the MAPK module is a negative regulator, the “two-component system,” comprised of three sequentially acting kinases including Sln1, a plasma membrane bound histidine/aspactyl kinase, Ypd1, a histidine kinase, and Ssk1, an aspartyl kinase (25, 27, 28). These kinases negatively regulate two MEKKs called Ssk2 and Ssk22 (29). There is also a positive regulator upstream of the MAPK module called Sho1 which activates Pbs2 directly (29). The model for activation of this pathway is as follows. Osmotic

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, MAP/ERK kinase; JNK/SAPK, Jun-N-terminal kinase/stress-activated protein kinase; PP2A, type-2A protein phosphatase; HP1, protein-tyrosine phosphatase; HOG, high osmolarity glycerol; Hog1-Tyr(P), Hog1-phosphotyrosine; GST, glutathione S-transferase; kb, kilobase pairs; PCR, polymerase chain reaction; HA, hemagglutinin epitope; PNPP, p-nitrophenyl phosphate; PAGE, polyacrylamide gel electrophoresis; bp, base pairs(s); 5-FOA, 5-fluoroorotic acid; CEN, centromere.
media refers to YPD, and high osmolarity media refers to YPD containing 0.9 M sodium chloride unless otherwise noted.

**Isolation and Deletion of PTP3—PTP3 was isolated as a negative regulator of the HOG pathway by selecting for plasmids from a yeast genomic library that suppressed lethality of the snl1Δ strain, IMY101.** This strain was transformed with a yeast genomic library based in the vector YEp13 (37) (American Type Culture Collection). Transformants capable of vigorous growth on 5-FOA were identified, and the plasmid DNA was isolated using standard methods. In addition to plasmids bearing PTP2, a different plasmid, pAF12, bearing an 8-kb insert was isolated multiple times. Deletion of a SpbI fragment or a EcoRI fragment from pAF12 identified regions of the strain critical for suppression. Sequencing adjacent to the SpbI site identified an open reading frame, Yer075p, sequenced by the genome project. We call this gene PTP3.

A strain bearing a deletion of the PTP3 gene was produced by transformation of a wild type diploid, DF5, with a PTP3 deletion construct. To produce the ptp3::TRP1 allele, PCR was used to introduce a BamHI site 275 bp upstream of the start site and a SmaI site 118 bp downstream of the start codon, generating a 400-bp fragment corresponding to the 5’ end of the gene. A fragment corresponding to the 3’ end of the gene was produced using PCR to generate a SmaI site 2644 bp downstream of the start site and an EcoRI site 985 bp downstream of the stop codon. Both fragments were simultaneously ligated into pUC19 digested with BamHI and EcoRI to generate the plasmid pUC19. This plasmid was digested with SmaI, and a 550-bp blunt-ended EcoRI-BglII fragment of TRP1 (38) was ligated to generate pUC19-tp3::TRP1. This plasmid was digested with EcoRI and BamHI and transformed into DF5, and Trp+ transformants were selected. Southern analysis identified transformants bearing the deletion allele at the PTP3 locus (39). Briefly, genomic DNA was digested with XbaI and probed with the 1-kb Smal-EcoRI fragment corresponding to the 3’ end of the PTP3 gene. A 4-kb fragment corresponding to the ptp3::TRP1 allele integrated at the correct locus, and a 5.1-kb fragment corresponding to the wild type PTP3 locus was detected in several transformants. Dissection of ptp3::TRP1/PTP3 heterozygous diploid strains resulted in a 2:2 segregation of Trp+ to Trp− spore clones.

**Mutagenesis of PTP2 and PTP3—PTP2 and PTP3 were mutagenized by PCR-based methods. To produce the mutant allele, PTP2-C666S, where Cys-666 is mutated to Ser, the oligonucleotide, 5'-GGACCATGCAGAAGATTGCATTAA-3', containing the underlined mutation was paired with a second oligonucleotide upstream of this site, 5'-GGGACATGGCATTCTGAGAGATCC-3'. To produce PTP2-C666A, where Cys-666 is mutated to Ala, the oligonucleotide, 5'-CACCTGGAGAAGCATGAGAGATC-3', containing the underlined mutation was paired with the second oligonucleotide described above. In each case the 707-bp PCR product was digested with PstI at two naturally occurring sites and cloned into pUC19. This mutagenized fragment, when completely sequenced, identified only those mutations introduced by the mutagenic oligonucleotide.** PTP2-C666S or PTP2-C666A was expressed in a low copy CEN-based plasmid bearing the TRP1 gene, by substituting the mutagenic PstI fragment for the wild type PstI fragment in the plasmid pHS2 (22), generating pPtp2-C666S and pPtp2-C666A. PTP2-C666S and PTP2-C666A were expressed in a high copy 2μ-based plasmid, also bearing the TRP1 gene, by cloning PvuII fragments from these plasmids into YEplac112 (40). To produce the mutant allele PTP2-C670A the mutagenic oligonucleotide, 5'-GTTTCT-GCAAGGCGTGAGAAGAC-3', bearing the underlined mutations and a second oligonucleotide corresponding to a region in the 3’-flanking sequence of the gene, 5’-CCCAAGCTTATAGCGAAAAATTACG-3’, were used to produce a 450-bp PCR product that was ligated together with wild type fragments from PTP2 into YEplac112. To produce the PTP2-C666A,C670A double mutant, fragments from each mutant were cloned into YEplac112.

The catalytic residue, Cys-804, in Ptp3, was mutated to Ala using similar methods. PTP3-C804A was produced by PCR using an oligonucleotide bearing two mutations, 5’-CAAGAAGACCTTCCTTCCACAC-CCTGCGAACAGATGACAAAAATTG-3’, which was paired with a second primer upstream of this site, 5’-GAGACGTATTTGAGTGCAGTC-3’, and cloned into pUC19. This PCR generated a 1.4-kb PCR product that was digested with XbaI alone or at naturally occurring sites and, together with a 647-bp BamHI fragment from the wild type PTP3 gene, cloned into pUC19. The 2-bp PstI-XbaI fragment together with a 1.5-kb XbaI-EcoRI fragment from the wild type PTP3 gene were simultaneously ligated into YEplac181, a 2μ-based plasmid bearing the LEU2 gene (40). Sequencing identified only two mutations corresponding to those introduced by the mutagenic oligonucleotide.

**Construction of PTP2, PTP3, and HOG1 Expression Plasmids—**

**Protein Tyrosine Phosphatases Inactivate the HOG Pathway**

**FIG. 1. The osmotic stress response pathway in S. cerevisiae.** In response to osmotic stress, the dephosphorylated forms of Sln1, Ypd1, and Sk1 activate the MEKKs, Sks2 and Sks22, the MEK, Pbs2, and the MAPK, Hog1. Activation of Hog1 requires phosphorylation of Thr-174 and Tyr-176 in the activation loop. Ptc1 is a negative regulator of this pathway that encodes a type 2C Ser/Thr phosphatase. Its substrate in this pathway is not known. PTP2 and PTP3 encode protein-tyrosine phosphatases that inactivate the pathway by acting on Hog1.

Osmotic shock inactivates the two-component system, resulting in activation of the MEKKs that in turn activate the MAPK module. Osmotic shock activates Sho1 which directly activates Pbs2 via an SH3 domain. Osmotic shock inactivates the two-component system, resulting in activation of the negative regulator, Sln1, constitutively activates the HOG pathway and is lethal on rich media and grows extremely poorly on less rich, synthetic media (27). Using this phenotype, we identified negative regulators as genes which, when over-expressed from a multicopy plasmid, restore viability. In addition to PTP2, we identified a second gene, PTP3, that inactivates this pathway by targeting Hog1. PTP2 and PTP3 are transcriptionally regulated; activation of the HOG pathway induces expression of PTP2 and PTP3, providing a mechanism for adaptation to signal.

**MATERIALS AND METHODS**

**Strains, Media, and Genetic Techniques—**To identify suppressors of the HOG pathway, a yeast genomic library was transformed into IMY101, a strain bearing a SLN1 deletion, sln1Δ::HIS3, and a low copy, CEN-based plasmid containing the wild type SLN1 gene and the URA3 gene (27). Yeast transformations were performed as described by Dohmen et al. (30). To follow the level of Hog1-Tyr(P), haploids derived from DF5 (31) were transformed with 2 μm HOG1, a multicopy 2μ-based plasmid bearing HOG1 and TRP1, kindly provided by M. Gustin (28). The strains bearing 2 μm HOG1 are BBY48, a wild type strain (32), IMY21a, a ptp2Δ strain (22), HF2, a ptp2Δ strain, and HPY6, a ptp2 ptp3Δ strain. For biochemical analysis of Ptp2 and Ptp3, glutathione S-transferase (GST)-PTP fusion proteins were expressed in RS334, kindly provided by R. Scalfani (33). To test whether the GST-PTP fusions were functional in vivo, the pGSt-PTP plasmids (see below) were transformed into TGY1, which bears the SLN1 deletion, sln1Δ::HIS3, and pSLN1::URA3. TGY1 is derived from JDG1, a galactose-inducible strain (34). A strain bearing a disruption of the PTC1/TPP1 gene was produced by transformation of DF5 with the tpd1::LEU2-Δ allele, kindly provided by J. Broach (35). The tpd1::LEU2-Δ/TPD1 diploids were sporulated and tetrads dissected, resulting in a 2:2 segregation of wild type and temperature-sensitive spore clones as reported (35). Media to culture yeast and bacteria were produced essentially as described by Sherman et al. (36). Standard rich media refers to YPD, and high osmolarity media refers to YPD containing 0.9 M sodium chloride unless otherwise noted.
and Ptp3 were expressed as fusions to GST using pEgK(CT), a 2-μm-based vector bearing the URA3 gene and GST under regulation of the GAL1/10 promoter (41). The plasmid pGST-PTP2 was constructed by introducing a BamHI site just upstream of the PTP2 start codon using PCR. The oligonucleotide, 5'-GGGATCCGATGATCGAGTCACG-ACGAC3' (underlined sequence) was cloned into pGEM-T Easy vector, 5'-GGCCGA-TATCCTTAGACATGG-3', corresponding to an EcoRV site 320 downstream of the start codon. The fragment was produced by PCR and was digested with BamHI and EcoRV and, together with wild type PTP2 fragments from the plasmid pH65.7 (22), cloned into pEG(CT). The plasmid pGST-PTP3 was constructed by introducing a BamHI site just upstream of the start codon using the same upstream oligonucleotide and the 3'-end oligonucleotide was paired with 5'-GGGATCCGATGATCGAGTCACGACGAC3', corresponding to a naturally occurring HindIII site 386 bp downstream of the start codon. The PCR product was digested with BamHI and HindIII and, together with fragments from the wild type PTP3 gene obtained from pAF12, cloned into pEG(CT). Mutants PTP2-C666S and PTP3-C804A were also fused to the carboxyl terminus of GST by similar methods. The plasmid pHOG1-HA, expresses HOG1 tagged at its carboxyl terminus with two repeats of the hemagglutinin epitope (Hog1-HA) under regulation of the CUP1 promoter in the vector Yep151. A HindIII site was engineered upstream of the HOG1 start codon, and a NoIs site was substituted at the stop codon to generate the fusion. A 400-bp BamHI-EcoRI fragment containing the CUP1 promoter was inserted upstream of the start codon. The Hog1-HA fusion protein is functional, since it complements the osmosensitivity of a hog1Delta strain.

**Assay for Phosphatase Activity—**The phosphatase activity of Ptp2 and Ptp3 was tested as follows. RS334 carrying pGST-PTP2 or pGST-PTP3 was grown in synthetic media lacking uracil and containing 2% galactose. Cells from 250 ml of culture grown to ∼1.0 unit (A600nm) were harvested and homogenized by glass beading in lysis buffer, 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl2, 0.2% Triton X-100, 0.1% 2-mercaptoethanol, with protease inhibitors (leupeptin, pepstatin A, antipain, aprotinin, and chymostatin each at 20 μg/ml) and 1 mM phenylmethylsulfonyl fluoride. The lysate was centrifuged at high speed for 10 min at 4 °C, and the supernatant was incubated with 45 μl of a 1:1 slurry of glutathione-Sepharose beads (Pharmacia Biotech Inc.) for 1.5 h at 4 °C. The beads were washed 3 × with lysis buffer, followed by 3 washes with lysis buffer containing 150 mM NaCl, and finally 3 × with lysis buffer containing 300 mM NaCl. Two proteins of ~112 kDa corresponding to GST-Ptp2 and ~125 kDa corresponding to GST-Ptp3 were found in these preparations as detected by SDS-PAGE and immuno-blotting with anti-GST antibody (Pharmacia) or by silver staining. These proteins were absent from RS334, which carries pEG(CT), and were found in these preparations as detected by SDS-PAGE and immunoblotting with anti-HA antibody (12CA5, Babco). Cells in exponential growth phase were osmotically shocked by the addition of an equal volume of media containing 0.8 M sodium chloride. Cells were harvested and homogenized by glass beading in lysis buffer containing 100 μM sodium orthovanadate and 50 μM β-glycerophosphate. Lysates were boiled with sample buffer, separated in SDS-PAGE gels and transferred to nitrocellulose membranes (Millipore) in 20% methanol, 25 mM Tris, 0.2 M glycine, 0.01% SDS, using a Genie transfer apparatus (Idea Scientific). The blot was blocked in TNST, 20 mM Tris, 0.15 M NaCl, 0.01% Tween, containing 1% bovine serum albumin for 1 h and incubated with primary antibody (PY20, ICN) at a dilution of 1:1000 in TNST for 1 h. After washing, rabbit anti-mouse alkaline phosphatase-conjugated antibody (Promega) was added at a dilution of 1:7500 in TNST for 30 min. The blot was washed with TNST, and the immunoreactivity was visualized using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (42) (Promega).

**RNA Analysis—**Yeast grown to ~1 unit, A600nm, in YPD were untreated or treated as shocked for 10 min by the addition of YPD containing with an equal volume of NaCl. These were harvested by centrifugation, and the RNA were prepared by freezing in phenol and SDS (43). Total RNA was electrophoresed in formaldehyde-containing agarose gels, and hybridization was performed by standard methods (39). The blot was hybridized with 32P-labeled probes to PTP2, PTP3, GPD1, and TUB1. A 750-bp PstI fragment internal to the PTP2 open reading frame, a 1.1-kb ClaI fragment internal to the PTP3 open reading frame, a ~1.1-kb BglII-ClaI fragment from TUB1, and a 814-bp SalI fragment from GPD1, containing 352 bp of the GPD1 open reading frame and 462 bp of upstream sequence, were used to produce 32P-labeled probes (39).

**RESULTS AND DISCUSSION**

**Identification of PTP3 as a Negative Regulator of the HOG MAPK Pathway—**Protein phosphatases that negatively regulate the S. cerevisiae HOG pathway were isolated by a genetic approach. A selection was devised using a sln1Delta strain whose severe growth defects are due to constitutive activation of the HOG MAPK cascade (25, 27). Since this growth defect can be alleviated by mutational inactivation of members of the MAPK cascade, overexpression of negative regulators should also suppress lethality. Such negative regulators should include protein phosphatases that inactivate the MAPK module. To identify such regulators, we used the strain, IMY101, lacking chromosomal copies of both SLN1 and URA3 and carrying both of these genes on a single plasmid, pSLN1-URA3 (27). Because this strain requires pSLN1-URA3 for viability, it is necessarily UraΔ and therefore unable to grow on media containing 5-FOA, which selects against URA3-expressing cells. Overexpression of negative regulators should allow survival of the sln1Delta strain in the absence of pSLN1-URA3 and thus growth on 5-FOA. This selection yielded two protein-tyrosine phosphatases, Ptp2, and a new gene, Ptp3, which, when overexpressed, inactivate the HOG pathway.

**Ptp2 and Ptp3 Show Differences in Their Ability to Inactivate the HOG Pathway—**To begin examining why two protein-tyrosine phosphatases regulate the HOG pathway, the effects of altering the level of expression of PTP2 and PTP3 were examined. Although both PTP2 and PTP3 suppress the severe growth defects of the sln1Delta strain when expressed from a multicopy 2-μm-based plasmid, only PTP2 suppressed the growth defect when expressed from a low copy CEN-based plasmid. Thus PTP2 may have a greater effect on the HOG pathway than PTP3. To test whether deletion of PTP2 would have a greater impact on the HOG pathway than deletion of PTP3, strains lacking either or both PTPs were constructed. No obvious differences were observed between ptp2Delta, ptp3Delta, ptp2Delta ptp3Delta, and wild type strains grown under standard conditions or in high osmolality media where the HOG pathway would be activated. Differences were observed between ptp2Delta and ptp3Delta strains, however, when combined with the sln1Delta mutation. The sln1Delta strain exhibits severe growth defects on synthetic media due to HOG pathway hyperactivation. If the role of these PTPs is to inactivate the HOG pathway, then their deletion should adversely affect the sln1Delta strain. The sln1Delta ptp2Delta double mutant is lethal on synthetic media, whereas the sln1Delta ptp3Delta strain grew as well as the sln1Delta strain on synthetic media (data not shown). These results suggest that the HOG pathway is more acutely affected by PTP2 than by PTP3.

**Ptp2 and Ptp3 Are Phosphatases Whose Activity Is Important for Inactivating Hog1—**Analysis of the primary sequence of Ptp2 and Ptp3 shows they are similar in structure, having a novel amino-terminal domain fused to a carboxy-terminal protein-tyrosine phosphatase (PTP) domain. The amino-terminal domains of Ptp2 and Ptp3 show two small regions of similarity...
to each other and to PyP1 and PyP2, which regulate an osmotic stress response pathway in *S. pombe*. One of these regions is also similar to PAC1 (44, 45), a vertebrate dual specificity phosphatase, which shows greater activity toward the vertebrate homolog of Hog1, p38, and ERK than to JNK/SAPK (12). The PTP domains, like those of the *S. pombe* Ptp2, are also similar to PAC1 (44, 45), a vertebrate dual specificity phosphatase, which shows greater activity toward the vertebrate homolog of Hog1, p38, and ERK than to JNK/SAPK (12).

Protein Tyrosine Phosphatases Inactivate the HOG Pathway

The requirement for Ptp2 and Ptp3 phosphatase activity was tested in vivo by determining whether the mutant PTPs could suppress the severe growth defect of the *sln1Δ* strain. The mutants Ptp2-C666S and Ptp2-C666A expressed from low copy CEN-based plasmids and the mutant Ptp3-C804A expressed from a multicopy 2-µm-based plasmid were not able to suppress the severe growth defects of the *sln1Δ* strain as well as their wild type counterparts (Fig. 3). Thus the phosphatase activity of Ptp2 and Ptp3 is required for inactivation of the HOG pathway. Interestingly, the Ptp2-C666S and Ptp2-C666A mutants retained some ability to suppress the growth defect of the *sln1Δ* strain. Expression of Ptp2-C666A from a low copy plasmid allows the *sln1Δ* strain to grow better than controls with empty vector (Fig. 3). Ptp2 and Ptp3 differ from other PTPs in yeast and vertebrates (Fig. 2) (8). The PTP domain of Ptp2 is most similar to Ptp3 and also shows strong similarities to other PTPs in yeast and vertebrates (Fig. 2) (8).

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Ptp2 and Ptp3 Regulate Hog1 in Vivo—If Ptp2 and Ptp3 dephosphorylate Hog1-Tyr(P) in vivo, strains lacking these phosphatases should show defects in their ability to dephosphorylate Hog1-Tyr(P). The following results indicate that Ptp2 and Ptp3 have two roles in the cell. One role is to maintain a low basal level of Hog1-Tyr(P) when cells are grown under standard conditions. A second role is in adaptation. The basal level of Hog1-Tyr(P) is highest in the ptp2Δ and ptp3Δ double mutant and was also elevated in the ptp2Δ mutant (Fig. 5). Low levels of Hog1-Tyr(P) were detected in the ptp3Δ strain, and none was visible in wild type. These results are interesting because Ptp2 and Ptp3 act as negative regulators of the HOG pathway, but do not exhibit the severe growth defects associated with Hog1 hyperactivation, as seen, for example, with mutations that inactivate SLN1 (25).

The most likely explanation for the lack of growth defects in ptp2Δ and ptp3Δ mutant strains is that Ptp2, and possibly Ptp3, specifically dephosphorylate phosphotyrosine but not phosphothreonine in the phosphorylation lip of Hog1. This might result if in ptp null strains, the level of Hog1-Tyr(P) is high, but the level of threonine phosphorylation is low, and as a result, Hog1 is not hyperactivated. Support for this hypothesis comes from the observation that deletion of a type 2C Ser/Thr phosphatase, PTC1, together with PTP2, produces a severe synthetic growth defect (47). PTC1 has been identified as a negative regulator of the HOG pathway, but its substrate is not known. If Ptc1 mediates dephosphorylation of phosphotyrosine in Hog1, the synthetic growth defect of ptc1Δ might be explained by hyperphosphorylation of Hog1 at both Thr and Tyr residues in the phosphorylation lip. To test this idea we deleted HOG1 or PBS2 in the ptc1Δ background. Both ptc1Δ and pbs2Δ strains grew as well as the ptc1 mutant (data not shown), indicating that the severe synthetic growth defect of the ptc1Δ strain is due to hyperactivation of the HOG pathway. Ptc1 inactivation of Hog1 may be direct, via dephosphorylation of Hog1-Thr(P)-174, or indirect, via dephosphorylation of Pbs2 or other upstream activators. Similar tests performed with PTP3 demonstrated no interaction with PTC1; the double mutant ptc1Δ ptp3Δ showed no synthetic growth defect (data not shown). These results suggest that Ptp2 has a greater role than Ptp3 in maintaining the low level of Hog1-Tyr(P) under standard growth conditions.

A second role for these PTPs is in adaptation. When wild type

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2 L. Freeman-Cook and I. M. Ota, unpublished observations.
cells are osmotically shocked Hog1-Tyr(P) levels increase rapidly, reaching maximal levels by ~5 min. This is followed by a rapid decrease in Hog1-Tyr(P) to nearly basal levels by 30 min (Fig. 5). If PTPs are responsible for this rapid decrease, strains lacking PTPs should dephosphorylate Hog1-Tyr(P) more slowly than wild type. Cultures of exponentially growing wild type and ptp2Δ, ptp3Δ, or ptp2Δ ptp3Δ strains were exposed to continuous osmotic stress, and at various times the level of Hog1-Tyr(P) was determined by SDS-PAGE and immunoblotting. Upon osmotic shock, Hog1-Tyr(P) increased in all strains, but the rates of Hog1-Tyr(P) dephosphorylation differ. The rate of dephosphorylation is most rapid in wild type and nearly as fast in the ptp3Δ strain (Fig. 5). Dephosphorylation of Hog1-Tyr(P) is significantly slower in the ptp2Δ strain and is dramatically slower in the ptp2Δ ptp3Δ double mutant (Fig. 5). Thus strains lacking PTP2, or PTP2 and PTP3, failed to dephosphorylate Hog1-Tyr(P) as well as wild type. One interesting feature of these data is that deletion of PTP3 had little effect on the rate of Hog1-Tyr(P) dephosphorylation, yet the ptp2Δ ptp3Δ had a synergistic effect, slowing Hog1-Tyr(P) dephosphorylation more than would be expected by the sum of each mutant alone. This effect could be explained if Ptp3 has other roles in addition to regulating Hog1 directly.

PTP2 and PTP3 Expression Are Induced by Osmotic Stress and Are Dependent upon Hog1—The results above indicate that Ptp2 and Ptp3 are involved in adaptation. Because the state of HOG pathway activation is sensitive to the level of PTP2 or PTP3 expression, one mechanism of adaptation might involve induction of PTP transcripts in response to osmotic stress. To test this idea, an exponential culture of a wild type strain was untreated or exposed to osmotic shock for 10 min at 30 °C. Total RNA was examined by Northern analysis. The level of PTP2 transcript increased ~2–3-fold, whereas PTP3 transcript increased ~5-fold following osmotic shock (Fig. 6). GPD1, encoding glycerol 3-phosphate, increased substantially as described previously (48) and TUB1 decreased slightly. Total RNA prepared from a hog1Δ strain that was untreated or exposed to osmotic stress showed no significant increases in the level of PTP2 or PTP3. The GPD1 transcript is still induced in the hog1Δ strain but to a much lesser degree than in wild type (48). Thus the HOG pathway is required for induction of PTP2 and PTP3 transcripts in response to osmotic shock, suggesting that activation of the HOG pathway triggers a negative feedback loop to inactivate the pathway.

Induction of PTP2 is likely to involve promoter stress elements also present in other genes regulated by the HOG pathway (49). Although the PTP2 promoter contains two exact matches to the stress elements, C₃T, PTP3 has none. Thus PTP3 induction may occur through other elements. Msn2 and Msn4, two zinc finger proteins, originally identified as suppressors of Snf1, a kinase involved in glucose sensing (50), have been shown to bind the stress elements in vitro, acting as transcriptional activators (51, 52). Msn2 and Msn4, however, are unlikely to be the only mediators of Hog1-activated transcription since msn2Δ msn4Δ strains, unlike hog1Δ, grow as well as wild type on media containing 0.8 M NaCl (51). Thus expression of PTP2 may involve these activators as well as others. MAPK pathway-induced expression of protein phosphatases is likely to be a general mechanism of adaptation. Expression of MSG5, a gene encoding a dual specificity phosphatase in S. cerevisiae, is induced by activation of the pheromone response pathway, and pyp2Δ, a gene encoding a PTP in S. pombe, is induced by an osmotic stress response pathway (15, 19, 20). Strains lacking pyp1Δ or pyp2Δ show a decreased rate of Sty1/Spc1-Tyr(P) dephosphorylation (19–21). Whether transcriptional activation of these genes is required for adaptation is not known.

In summary, we have described two protein-tyrosine phosphatases that regulate the HOG pathway in S. cerevisiae by targeting the Hog1 MAPK. Both PTP2 and PTP3 express phosphatase activity, and this activity is required in vivo for pathway inactivation. Mutant Ptp2 and Ptp3 bind Hog1 in vitro, and strains lacking PTP2 and PTP3 show elevated levels of Hog1-Tyr(P), strongly suggesting they inactivate this pathway by dephosphorylating Hog1-Tyr(P). Why two PTPs are needed to regulate Hog1 is not clear. We find, however, that the activity of the HOG pathway is more sensitive to PTP2 than to PTP3. Expression of PTP2 from a low copy plasmid is sufficient to suppress the growth defects of the sln1Δ strain, whereas multicopy expression of PTP3 is required for suppression. This is corroborated by synthetic lethality of the ptp2Δ sln1Δ strain but not the ptp3Δ sln1Δ strain, the synthetic growth defect of the ptp2Δ ptc1Δ strain but not ptp3Δ sln1Δ strain, and differences in the level of Hog1-Tyr(P) in ptp2Δ versus ptp3Δ strains. The greater induction of PTP3 seen in response to osmotic shock compared with PTP2 does not compensate for the differ-
ences between these PTPs since the ptp2Δ strain dephosphorylates Hog1-Tyr(P) at a substantially slower rate compared with the ptp3Δ strain. Further investigation of the regulation of these PTPs, possibly via their posttranslational modification or subcellular localization, should contribute to a better understanding of the roles of these PTPs in the HOG pathway.

Osmotic stress response pathways operate in S. cerevisiae, S. pombe, and mammals. Thus far, Ptp2, Ptp3, and the S. pombe Pyp1 and Pyp2 are the only PTPs known to regulate MAPK signaling pathways. These PTPs show sequence similarities not found in other PTPs, making it tempting to speculate that they confer specificity for osmoregulatory MAPKs. The PTP signaling pathways. These PTPs show sequence similarities to the yeast Hog1, is inactivated by the vertebrate dual specificity phosphatases, MKP-1, PAC1, and M3/6 (12, 46). Whether ptyl1 and ptyl2 is sufficient to activate Hog1-Tyr(P) at a substantially slower rate compared

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