Heat Stress Activates the Yeast High-Osmolarity Glycerol Mitogen-Activated Protein Kinase Pathway, and Protein Tyrosine Phosphatases Are Essential under Heat Stress

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The yeast high-osmolarity glycerol (HOG) mitogen-activated protein kinase (MAPK) pathway has been characterized as being activated solely by osmotic stress. In this work, we show that the Hog1 MAPK is also activated by heat stress and that Sho1, previously identified as a membrane-bound osmosensor, is required for heat stress activation of Hog1. The two-component signaling protein, Shn1, the second osmosensor in the HOG pathway, was not involved in heat stress activation of Hog1, suggesting that the Sho1 and Shn1 sensors discriminate between stresses. The possible function of Hog1 activation during heat stress was examined, and it was found that the hog1Δ strain does not recover as rapidly from heat stress as well as the wild type. It was also found that protein tyrosine phosphatases (PTPs) Ptp2 and Ptp3, which inactivate Hog1, have two functions during heat stress. First, they are essential for survival at elevated temperatures, preventing lethality due to Hog1 hyperactivation. Second, they block inappropriate cross talk between the HOG and the cell wall integrity MAPK pathways, suggesting that PTPs are important for maintaining specificity in MAPK signaling pathways.

Eukaryotes respond to a variety of stresses, including osmotic stress, heat stress, and radiation, by activating mitogen-activated protein kinase (MAPK) pathways. In vertebrates, two such stress response pathways have been identified as containing the MAPKs c-Jun NH2-terminal kinase and p38 (16, 42). In the yeast Saccharomyces cerevisiae, two MAPK pathways regulate the response to stress. The high-osmolarity glycerol (HOG) pathway, containing the MAPK Hog1, responds to osmotic stress, while the cell wall integrity pathway, containing the MAPK Mpk1, is activated by heat stress and hypo-osmotic stress (6, 10, 14). One aspect of stress response pathways that is not well understood is how stress is sensed. The S. cerevisiae HOG pathway is thought to sense osmotic stress via two membrane-bound regulators, each of which regulates a downstream MAPK cascade (Fig. 1). One branch is the two-component system containing Shn1, a plasma membrane-localized histidine kinase response regulator protein; Ypd1, a histidine kinase; and Ssk1, a second response regulator protein (20, 28, 30, 33). Genetic and biochemical studies indicate that Shn1-Ypd1-Ssk1 negatively regulates the downstream MAPK cascade comprising the MEKKs (MAPK/extracellular signal-regulated kinase [ERK] kinase kinases) Ssk2 and Ssk22, the MEK (MAPK/ERK kinase) Pbs2, and the MAPK Hog1 (20, 33). The second branch contains membrane-bound Sho1, which contains an SH3 domain through which it interacts with Pbs2 (18, 35). Sho1 transduces signals via the small G protein Cdc42, the p21-activated kinase Ste20, the novel protein Ste50, and the MEKK Ste11 (27, 32, 34, 35).

Another aspect of MAPK signaling pathways that is not well understood is their negative regulation. Protein phosphatases play a key role; however, their functions have not been intensively examined. The importance of protein phosphatases in the negative regulation of MAPK pathways is evident. For example, mutation of the Drosophila puckered gene encoding a protein tyrosine phosphatase (PTP) that inactivates c-Jun NH2-terminal kinase results in defects in dorsal closure during embryogenesis (21). In Schizosaccharomyces pombe, overexpression or deletion of PTPs that inactivate the stress-activated MAPK Spc1 results in cell cycle defects (37), and in S. cerevisiae, deletion of protein phosphatases that inactivate Hog1 is nearly lethal due to hyperactivation of this pathway (13, 19).

Three different classes of protein phosphatases have been identified that inactivate MAPKs in yeasts and in vertebrates. Since MAPKs require dual phosphorylation of a Thr residue and a Tyr residue in the activation loop for full activity, dual-specificity phosphatases (DSPs), capable of dephosphorylating both phosphothreonine and phosphotyrosine residues, PTPs specific for phosphotyrosine, and Ser/Thr phosphatases specific for phosphothreonine, and Ser/Thr phosphatases specific for phosphothreonine and phosphotyrosine residues have been found as MAPK regulators. In S. cerevisiae, six MAPK pathways regulate a variety of biological responses (10), and a DSP, PTPs, and type 2C Ser/Thr phosphatases (PF2Cs) regulate the MAPKs. The DSP Msg5 inactivates the MAPK Fus3 in the pheromone response pathway (7) but not other MAPKs. Two PTPs, Ptp2 and Ptp3, inactivate Hog1 (Fig. 1), Mpk1, and Fus3 with different specificities (13, 23, 44, 45). In addition to dephosphorylating Hog1, the PTPs in the HOG pathway modulate Hog1 subcellular localization by binding Hog1 (22). Dephosphorylation of the phosphothreonine residue in Hog1 is performed by the PF2Cs Ptc1 (40) and Ptc2 and Ptc3 (C. Young, J. Mapes, J. Hanne-

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HOG/Heat Stress Pathway

FIG. 1. Osmotic stress versus heat stress activation of the HOG pathway. The HOG pathway is regulated by two membrane-bound proteins, Snl1 and Sho1. Osmotic stress activates the MAPK Hog1 via the Snl1 and Sho1 osmosensors. The two-component system, Snl1-Ypd1-Ssk1, negatively regulates the MEKs Sks2 and Ssk22, while Sho1, Ste20, Cdc42, and Ste50 positively regulate the MEKK Ste11. Once the MEKs are activated, they phosphorylate and activate the MEK Pbs2 and the MAPK Hog1. In contrast to osmotic stress, heat stress activates Hog1 via the Sho1 branch, but not the two-component system. Two PTPs, Ptp2 and Ptp3, inactivate Hog1 by dephosphorylating the phosphotyrosine residue, while the PP2Cs Ptc1 and Ptc2 or Ptc3 dephosphorylate the phosphotyrosine residue in the activation loop.

man, S. Al-Zarban, and I. M. Ota, submitted for publication) (Fig. 1).

To uncover new functions of protein phosphatases, we examined the properties of phosphatase null strains in S. cerevisiae. We found that the strain lacking PTP2 and PTP3 was inviable under heat stress and that lethality was dependent upon HOG1. This analysis suggested that Hog1 was activated by heat stress; indeed, biochemical assays indicated that this was so. Surprisingly, the Sho1 branch but not the two-component branch of the HOG pathway mediated heat stress activation of Hog1. These studies show for the first time that the HOG pathway can respond to heat stress and suggest that stress sensors can discriminate between different stress signals. We also examined the role of PTP2 and PTP3 in the heat stress response. Since the HOG and cell wall integrity pathways are both activated by heat stress, it seemed possible that PTPs might be important for preventing cross talk between these pathways. Indeed, deletion of PTPs led to cross talk between the cell wall integrity MEK and the MAPK Hog1, indicating that PTPs are important for blocking signaling between MAPK pathways.

MATERIALS AND METHODS

Strains and genetic techniques. The strains used in this work are listed in Table 1. All strains were derived from the wild-type diploid, DF5, or its haploid disectors, BBY45 and BBY48 (2), unless otherwise noted. Fus3 was deleted from BBY46 to produce CMY13 (MATa::ura3-52::HIS3). The fus3Δ::LEU2 allele was obtained from pJB225 (a gift from E. Elion). SSK1 was deleted from BBY45 to produce IMY111 by using the sks1Δ::TRP1 allele from plasmid psk11TRP1, described below. SHO1 was deleted from BBY48 to produce CMY18 by using the sho1Δ::LEU2 allele from pSKsho1::LEU2, described below. IMY114 (ptp2Δ::LEU2) was produced by transforming BBY48 with the pht2Δ::LEU2 allele, described below. IMY108 (ptp2Δ::URA3) was produced by transforming BBY48 with the pht2Δ::URA3 allele contained in plasmid pMM211 (gifts from M. Gustin). Strain IMY117 (ptp3Δ::HIS3) was produced by transforming JD52 (8) with the deletion allele from pCM1 (23). Deletion of each of these genes, Fus3, Ssk1, Sho1, Ptp2, Ptp3, and Ptp2, was confirmed by Southern analysis or by PCR. Strains bearing these deletions in combination with others were produced by standard genetic methods.

To examine the role of PTP catalytic activity in HOG pathway, we expressed wild-type PTPs, catalytically inactive PTPs, and empty vectors were produced. HAY1 (ptp2Δ::HIS3 ptp1Δ::HIS3) was transformed with multicopy plasmids expressing wild-type PTPs, p112PTP2 (TRP1, 2μm) and p181PTP3 (LEU2, 2μm) (13); mutant PTPs, p112PTP2C66S (TRP1, 2μm) and p181PTP3C804A (LEU2, 2μm) (13); and empty vectors, Yeplac112 (TRP1, 2μm) and Yeplac181 (LEU2, 2μm) (9).

To examine the effect of deleting PTP2 and PTP3 on GPD1 expression, wild-type and ptp2Δ ptp3Δ strains were constructed that expressed β-galactosidase under the regulation of the GPD1 promoter. A yeast integrating plasmid, Yeplac112lacZ, was produced (see below), digested at a unique NotI site 353 bp from the GPD1 start codon, and transformed into D5 (2). Use of trans- sformants was selected, and Southern analysis was produced to confirm integration at the GPD1 locus. Heterozygous diploids bearing the GPD1::lacZ fusion were sporulated and dissected to produce haploid strain CMY15 (MATa::URA3::HIS3). To produce a ptp2Δ ptp3Δ strain expressing GPD1::lacZ, CMY15 and HFY6 (MATa::URA3::HIS3) were mated, diploids were sporulated, and tetrads were dissected. Use of His Trp spore clones were isolated as ptp2Δ::HIS3 ptp3Δ::TRP1 GPD1::lacZ::URA3 strain CMY16.

Plasmids. Plasmids carrying wild-type HOG1 fused to the hemagglutinin (ha) epitope (p181HOG1ha3) and catalytically inactive hog1K52M fused to the same epitope (p181HOG1K52Mha3) were constructed as follows. The HOG1 stop codon was substituted with a NotI restriction site by PCR, and an ~100-bp NotI fragment containing three repeats of the ha epitope (ha3) was ligated. A 1.5-kb SalI-KpnI fragment containing the 3' end of HOG1 fused to ha3 was ligated, together with a 1.1-kb SalI fragment containing the 5' end of the HOG1 gene, into the 2μm-based vector Yeplac181 (9). The resulting plasmid, p181HOG1ha3, complemented the osmotic stress sensitivity of a hog1Δ strain. The hog1K52M allele was produced by PCR with the mutagenic oligonucleotide 5′-CATTCAAGGCACGATGGTCCATGATG-3′ (the mutations are underlined). A 500-bp EcoRI fragment containing the mutation was substituted for the wild-type EcoRI fragment in p181HOG1ha3 to produce p181HOG1K52Mha3. This plasmid did not complement the osmotic stress sensitivity of a hog1Δ strain. Plasmids expressing wild-type PBS2 (p112PBS2) and catalytically inactive pbs2ΔK389M (p112PBS2K389M) were also produced. PBS2, contained in an ~3.4-kb genomic Cln1-Sac1 fragment, was ligated to the multicopy vector pRS424 (HIS3, 2μm) to produce p423PBS2. The ~3.4-kb Sac1-Suc1 fragment from p423PBS2 was cloned into the low-copy-number vector YCplac111 (LEU2, CEN/ARS) (9) to produce p112PBS2. The pbs2ΔK389M mutation was introduced by using a QuickChange site-directed mutagenesis kit (Stratagen). A 707-bp EcoRV fragment containing the K389M mutation was introduced for the wild-type fragment in p112PBS2 to produce p112PBS2K389M.

To delete SSK1, SHO1, and PTP2, the following plasmids were constructed. A plasmid containing the 5' and 3' flanking sequences of SSK1 (pSKsho1) was produced by PCR, and the TRP1 gene was inserted to produce pSS1::TRP1. To delete SHO1, a plasmid containing its 5' and 3' flanking sequences (pSskho1) was constructed by PCR, and the LEU2 gene, contained in a 2.2-kb Sac1-Sho1 fragment, was inserted to produce pSskho1::LEU2. The ptp2Δ::LEU2 allele was constructed by using plasmid pH4B.85 (29), which contains 5'- and 3'-flanking
sequences of PTP2. This plasmid was digested with EcoRV and ligated with the fragment described above containing LEU2.

To examine GPD1 expression, plasmid YlpGPD1::lacZ, a yeast integrating vector containing the GPD1 promoter fused to the lacZ gene (GPD1::lacZ), was constructed. An 813-bp BamHI-SalI GPD1 fragment, containing 462 bp upstream of the start codon and 351 bp downstream of the start codon, was ligated to yeast pRS315 (URA3, integrating vector) (25) to produce an in-frame fusion to LacZ. The GPD1 fragment was produced by PCR with oligonucleotides 5'-GGGATCCGACCATGGTCTCCTACTG-3' and 5'-GGAATTCCTAAAGCAGGTCATCGAAGCAATG-3'.

Immunoblotting. Heat stress activation of Hog1 was examined by using BBY48. Tyr-phosphorylated Hog1 (Hog1-P) was detected by using antiphosphotyrosine antibody (PV20; ICN) and dual phosphorylated Hog1 (phospho-p38 and phospho-tyrosine antibody; New England Biolabs, Beverly, Mass.) (40). To examine phosphorylation of p38, an isogenic ptp2Δ strain was examined. GPD1::lacZ and pVP7MKK1-386-expressing cells, exponential cultures of J52 (MATa trp1Δ ura3-52 his3-Δ200 leu2-3,112 lys2-801 63) 40, were treated with 5% DMSO for 10 min to activate (41).

### Table 1. Yeast strains

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*a All strains were derived from DF5 unless otherwise indicated.

*b Derived from JDS2.
and 15 μl of 0.1% sodium dodecyl sulfate (SDS) were added before vortexing. To this mixture was added 100 μl of 4-mg/ml o-nitrophenyl-β-D-galactopyranoside (Sigma), and the mixture was incubated at 30 °C for 10 min. The reaction was quenched by the addition of 500 μl of 1 M Na2CO3, and the A420 nm was measured.

RESULTS

Ptp2 and Ptp3 are necessary for survival under heat stress. Protein phosphatases inactivate MAPK signaling pathways, and their absence can result in poor growth due to constitutive activation of MAPKs (13, 19). While examining the phenotype of strains lacking PTPs, we found that a ptp2Δ ptp3Δ double mutant was unable to grow at 37 °C, although it was viable at 30 °C (Fig. 2A). A ptp2Δ single mutant showed a slight defect at 37 °C, while a ptp3Δ strain showed no defect (Fig. 2A); neither strain showed a defect at 30 °C. The temperature-sensitive phenotype of the ptp2Δ ptp3Δ and ptp2Δ strains could be due to a lack of induction of a heat stress response or, alternatively, to hyperactivation of the MAPKs that they inactivate. If the latter were true, then deletion of their MAPK substrates should alleviate ptp2Δ ptp3Δ temperature sensitivity. Since Ptp2 is known to strongly affect Hog1 dephosphorylation (13, 44), we first tested whether deletion of HOG1 could suppress the temperature-sensitive defect. A ptp2Δ ptp3Δ hog1Δ strain (CMY12) grew nearly as well as the wild type at 37 °C (Fig. 2A), and a ptp2Δ hog1K52M strain (CMY12 expressing the kinase-inactive mutant Hog1K52M from plasmid p181HOG1K52M) at 37 and 30 °C on selective medium. (C) Catalytic site mutant PTPs block ptp2Δ ptp3Δ temperature sensitivity. The wild type (JHY1 ptp2Δ ptp3Δ expressing the kinase-inactive mutant Hog1K52M from plasmid p181HOG1K52M) were grown on YPD medium at 37 and 30°C.

FIG. 2. Strains lacking the PTPs Ptp2 and Ptp3 are temperature sensitive due to Hog1 hyperactivation. (A) Growth of the wild-type and strains lacking PTPs, either alone or in combination with a HOG1 or a FUS3 deletion, was examined under heat stress. A wild-type strain (BBY48) and isogenic ptp2Δ ptp3Δ (HFY6b), ptp2Δ ptp3Δ hog1Δ (CMY12), ptp2Δ ptp3Δ fus3Δ (CMY14), ptp3Δ (HFY2), and ptp2Δ (IMY1b) strains were grown on standard rich medium, YPD, at 37 or 30°C for 3 days. (B) Hog1 kinase activity is necessary for ptp2Δ ptp3Δ temperature sensitivity. The growth of a ptp2Δ ptp3Δ HOG1 strain (CMY12 ptp2Δ ptp3Δ hog1Δ carrying plasmid p181HOG1) was compared to that of a ptp2Δ ptp3Δ hog1K52M strain (CMY12 expressing the kinase-inactive mutant Hog1K52M from plasmid p181HOG1K52M) at 37 and 30 °C on selective medium. (C) Catalytic site mutant PTPs block ptp2Δ ptp3Δ temperature sensitivity. The wild type (JHY1 ptp2Δ ptp3Δ expressing wild-type PTPs on multicopy plasmids p112PTP2 and p181PTP3), mutant ptp2C666S ptp3C804A (JHY1 carrying empty vectors YEplac112 and YEplac181) and mutant ptp2C666S ptp3C804A (JHY1 carrying phosphatase catalytic site mutants Ptp2C666S and Ptp3C804A on plasmids p112PTP2C666S and p181PTP3C804A, respectively) were grown on YPD medium at 37 and 30°C.
activation of Hog1 and Mpk1 could be responsible for ptp2Δ ptp3Δ temperature sensitivity. If so, then deletion of MPK1 should suppress the ptp2Δ ptp3Δ defect. For this test, the ptp2Δ ptp3Δ mpk1Δ strain was grown at 37°C on osmoremedial medium, which is supplemented with an osmotic stabilizer, 1 M sorbitol. It was necessary to do so, since mpk1Δ cells are inviable at 37°C due to a cell wall lysis defect unless grown on such medium (15). Although the mpk1Δ strain was viable at 37°C on this medium, the mpk1Δ ptp2Δ ptp3Δ strain was not (data not shown), suggesting that the temperature sensitivity of the ptp2Δ ptp3Δ strain was due primarily to heat stress activation of Hog1 and not Mpk1. We also tested whether BCK1, encoding the MEKK in the cell wall integrity pathway, could be involved in ptp2Δ ptp3Δ temperature sensitivity. However, the ptp2Δ ptp3Δ bck1Δ strain was still nonviable at 37°C on osmoremedial medium (data not shown). It should be pointed out that one limitation of these experiments was that the osmoremedial medium required to support the mpk1Δ ptp2Δ ptp3Δ and bck1Δ ptp2Δ ptp3Δ strains would result in the activation of Hog1. However, since the deletion of HOG1 largely suppressed the temperature sensitivity of the ptp2Δ ptp3Δ strain, Hog1 is primarily affected by heat stress in the phosphatase mutant.

Ptp2 and Ptp3 temperature sensitivity requires active Hog1. The results reported above suggested that ptp2Δ ptp3Δ temperature sensitivity is a result of Hog1 kinase hyperactivation. To test this notion, wild-type HOG1 was substituted with catalytically inactive hog1K52M. The ptp2Δ ptp3Δ hog1K52M strain grew well at 37°C (Fig. 2B), indicating that Hog1 kinase activity was necessary for the ptp2Δ ptp3Δ defect. It follows that ptp2Δ ptp3Δ temperature sensitivity is due to an inability to inactivate Hog1 by dephosphorylation of Hog1-pY. If so, then substitution of wild-type PTPs with the catalytically inactive mutants Ptp2C666S and Ptp3C804A (13) should also result in temperature sensitivity. Mutant PTPs or wild-type PTPs were expressed in a ptp2Δ ptp3Δ strain. As expected, the wild-type PTP2 PTP3 strain grew at 37°C, while the strain carrying an empty vector did not (Fig. 2C). The strain expressing mutant PTPs, however, grew as well as the wild type (Fig. 2C). The resistance of the strain with mutant PTPs is likely due to sequestration of activated Hog1. Mutant PTPs have been shown to bind Hog1 more effectively than wild-type PTPs (23, 44), sequester Hog1 in discrete subcellular compartments (22), and inactivate the HOG pathway when overexpressed (13). Therefore, Hog1 kinase activity is necessary, but not sufficient, for ptp2Δ ptp3Δ mutant temperature sensitivity.

The MEK Pbs2 and the Sho1 branch, but not the two-component system, are involved in heat stress activation of Hog1. Since many of the upstream components required for osmotic stress activation of Hog1 have been identified (Fig. 1) (18, 20, 27, 32, 33), we tested whether these same components could be involved in heat stress activation of Hog1. If so, then deletion of upstream regulators should suppress ptp2Δ ptp3Δ temperature sensitivity. Deletion of the MEK-encoding gene, PBS2, suppressed the ptp2Δ ptp3Δ defect (Fig. 3A), suggesting that Pbs2 is the primary MEK involved in the heat stress response. Since Pbs2 is thought to act as a scaffold, binding Sho1, Ste11, and Hog1 (31), its deletion could disrupt signaling by mechanisms other than direct inhibition of Hog1 phosphorylation. Therefore, PBS2 was substituted with catalytically in-active pbs2K389M. The ptp2Δ ptp3Δ pbs2K389M strain was viable at 37°C (Fig. 3B), indicating that Pbs2 kinase activity was necessary for ptp2Δ ptp3Δ temperature sensitivity.

We next tested whether the putative osmosensing proteins in this pathway could act as heat stress sensors. The two-component system, Sln1-Ypd1-Sxx1, and the novel protein, Sho1, are necessary for osmotic stress activation of Hog1 (18, 33). To test whether these proteins are also involved in heat stress activation of Hog1, the two-component system and SHO1 were deleted. SSK1 was deleted rather than SLN1, since removal of the latter is lethal (20, 30). Deletion of both SSK1 and SHO1 suppressed the temperature sensitivity of the ptp2Δ ptp3Δ strain, suggesting that both may mediate the heat stress response (Fig. 3C).

To test whether either or both of these proteins were involved, the ability of individual SHO1 and SSK1 deletions to suppress ptp2Δ ptp3Δ temperature sensitivity was examined. Deletion of SHO1 largely suppressed ptp2Δ ptp3Δ temperature sensitivity, while deletion of SSK1 had little effect (Fig. 3C), suggesting that Sho1, but not the two-component system, could be a heat stress sensor. This result was somewhat unexpected, as two-component signaling proteins in bacteria have been shown to act as heat stress sensors (17, 24, 26). To test whether this signal was transduced through other components in the Sho1 branch of the HOG pathway, the STE20, STE50, and STE11 genes were deleted. Each of the resulting strains, ste20Δ ptp2Δ ptp3Δ, ste50Δ ptp2Δ ptp3Δ, and ste11Δ ptp2Δ ptp3Δ, was no longer temperature sensitive (Fig. 3D), indicating that the heat stress signal was transduced through Sho1, Ste20, Ste50, Ste11, Pbs2, and Hog1.

Hog1 is activated by heat stress. The results reported above suggested two possibilities for heat stress activation of Hog1. First, such activation of Hog1 may occur only in a ptp2Δ ptp3Δ strain. The other possibility is that heat stress also activates Hog1 in the wild-type strain. Therefore, we examined Hog1 activation loop phosphorylation and Hog1 kinase activity in a wild-type PTP strain. Hog1 was rapidly phosphorylated and activated upon a shift from 23 to 39°C (Fig. 4A). Kinase activity increased ~4- to 5-fold (Fig. 4B), a modest activation compared to that seen with osmotic stress, which activates Hog1 ~25-fold (40). However, we believe that the heat stress activation of Hog1 was significant, since the MAPK Mpk1, required for growth at an elevated temperature and shown to be activated by heat stress (14), was activated ~2-fold with the same assay in our strain background (data not shown).

We next tested whether upstream regulators of the HOG pathway, Pbs2, Ssk1, and Sho1, were involved in heat stress activation of Hog1 when PTPs were present. Deletion of PBS2 or deletion of both SSK1 and SHO1 blocked heat stress-induced Hog1 activity (Fig. 4B). Deletion of SHO1 greatly inhibited heat stress activation of Hog1, while deletion of SSK1 had little effect (Fig. 4C). Thus, in agreement with the results of the phenotypic analysis, Pbs2 is the primary MEK mediating the heat stress response in this pathway, and Sho1, but not the two-component system, is required for heat stress activation of Hog1.

To test the role of the HOG pathway in heat stress, we compared the growth of wild-type and hog1Δ strains. Deletion of HOG1 led to one reproducible heat stress defect. The hog1Δ strain recovered more slowly from heat stress than wild type (Fig. 5). Both mutant and wild-type strains were grown at 23°C,
shifted to 39°C for 22 h, and allowed to recover at 23°C. The hog1Δ strain showed a reproducible growth lag relative to wild type after this treatment (Fig. 5). After an additional 10 h of recovery, the hog1Δ colonies were similar in size and number to wild type (data not shown). Therefore, the hog1Δ strain shows delayed growth in response to heat stress from which it is able to recover, suggesting Hog1 can facilitate recovery from heat stress.
Ptp2 and Ptp3 prevent hyperactivation of Hog1 during heat stress. The temperature sensitivity of the \( \text{ptp2}\Delta \text{ptp3}\Delta \) strain and its suppression by deletion of \( \text{HOGL} \) suggested that \( \text{ptp2}\Delta \text{ptp3}\Delta \) lethality is due to heat stress hyperactivation of Hog1. To examine this idea further, we assayed Hog1 kinase activity in the \( \text{ptp2}\Delta \text{ptp3}\Delta \) strain. We expected that the \( \text{ptp2}\Delta \text{ptp3}\Delta \) mutant would show greatly increased activation of Hog1 upon heat stress and/or an inability to inactivate Hog1 during prolonged heat stress. The latter would be consistent with a role for PTPs in adaptation, as shown for osmotic stress regulation of this pathway (13, 44). As described previously, the basal activity of Hog1 was elevated in the \( \text{ptp2}\Delta \text{ptp3}\Delta \) mutant (46). In our strain background, Hog1 kinase activity was elevated ~10-fold in the \( \text{ptp2}\Delta \text{ptp3}\Delta \) strain compared to the wild type in the absence of heat stress (Fig. 6A). Consistent with this result, the expression of \( \text{GPD1} \), a downstream target of the HOG pathway (1), was also upregulated (Fig. 6B). Increased Hog1 basal activity cannot be the cause of lethality, however, since the \( \text{ptp2}\Delta \text{ptp3}\Delta \) strain was viable at 23°C. Upon a shift to 39°C, Hog1 kinase activity increased in the \( \text{ptp2}\Delta \text{ptp3}\Delta \) mutant, to a level that was nearly sixfold higher than that in heat-treated wild-type cells. Similarly, \( \text{GPD1} \) expression was induced to a higher level in the \( \text{ptp2}\Delta \text{ptp3}\Delta \) strain (Fig. 6B), indicating that Hog1 activity affected downstream components. The \( \text{ptp2}\Delta \text{ptp3}\Delta \) strain showed no obvious defect during adaptation, since Hog1 kinase activity reached prestress levels after 15 min (Fig. 6A). Inactivation of Hog1 during adaptation was likely due to the activity of the PTPs Ptc1, Ptc2, and Ptc3, which inactivate Hog1 by dephosphorylating the phosphothreonine residue in the activation loop (Fig. 1) 40; Young et al., submitted). These results suggest that Hog1 activity, which starts at a significantly higher basal level in the \( \text{ptp2}\Delta \text{ptp3}\Delta \) strain, crosses a threshold upon heat stress which is lethal.

PTPs can prevent inappropriate activation of Hog1. Another potential role of MAPK phosphatases could be to prevent inappropriate cross talk between MAPK pathways. For example, since the cell wall integrity pathway is activated by heat stress and contains a MAPK cascade similar to that of the HOG pathway, the absence of PTPs could facilitate cross talk between these pathways. Such inappropriate cross talk could contribute to the Hog1 hyperactivation and lethality seen in the PTP null strain. One approach to test whether the cell wall integrity pathway contributes to Hog1 phosphorylation would be to delete components of the cell wall pathway. However,
this strategy could not be used, as strains lacking components of the cell wall pathway require a high-osmolarity environment for survival and high osmolarity itself activates Hog1. Therefore, we activated the cell wall pathway by using an inducible MEK allele driven from the GAL promoter (41). Overexpression of hyperactive MKK1-386 has been shown to induce a modest growth defect due to hyperactivation of its downstream target, Mpk1, in the presence of PTPs (23, 41). When PTPs were deleted, overexpression of MKK1-386 was lethal (Fig. 6C) (23). However, deletion of HOG1 suppressed lethality (Fig. 6C). These results suggest that Mkk1-386 activates Hog1 only when PTPs are absent. Indeed, immunoblotting with an antibody specific for dually phosphorylated Hog1 showed that Mkk1-386 activated Hog1 in the ptp2Δ ptp3Δ strain but not in the wild-type PTP strain (Fig. 6C). Furthermore, we found that MKK1-386 was lethal for the pbs2Δ ptp2Δ ptp3Δ strain (data not shown), suggesting that in the absence of PTPs, Mkk1-386 can directly activate Hog1 despite the lack of its normally required activator, the MEK Pbs2. Therefore, PTPs can protect Hog1 from inappropriate activation by the cell wall integrity MEK.

DISCUSSION

In this work, we found that heat stress activates Hog1 and that this effect was dependent on the Sho1 branch, but not the two-component system. Initially, we observed that a strain lacking PTP2 and PTP3, encoding two PTPs that inactivate Hog1, was inviable under heat stress. We showed that this defect was dependent on Hog1 activity, as its deletion or substitution with catalytically inactive Hog1K52M suppressed this defect (Fig. 2A and B). Furthermore, deletion of the upstream MEK Pbs2 or its replacement with catalytically inactive Pbs2K389M also suppressed this defect (Fig. 3A and B). These results implied that Hog1 was activated by heat stress and that the lack of PTPs led to Hog1 hyperactivation and lethality. Indeed, biochemical analysis showed that Hog1 was activated by heat stress in the wild type (Fig. 4A and B) and that it was hyperactivated in a ptp2Δ ptp3Δ strain (Fig. 6A).

We also examined the components of the HOG signaling pathway that were necessary for the heat stress activation of Hog1 and found that only one branch was required. Genetic data indicated that the heat stress signal was mediated by the Sho1 branch, as deletion of SHO1, STE20, STE50, and STE11 suppressed the ptp2Δ ptp3Δ temperature-sensitive defect (Fig. 1 and 3C and D). In contrast, deletion of SSK1 did not suppress this defect (Fig. 3C). Biochemical analysis corroborated the phenotypic data, as Hog1 kinase activity could not be activated by heat stress in a strain lacking Sho1 but could be activated as well as in the wild type when SSK1 was deleted (Fig. 4C). Therefore, heat stress activation of this pathway differs from osmotic stress and potentially oxidative stress, which can be mediated by the Sho1 and two-component signaling branches of this pathway (18, 20, 38).

The observation that the Sho1 branch, but not the two-component system, mediates the heat stress signal suggests that stress sensors do not necessarily respond to a feature common to all stresses but that they can discriminate between stress signals. This notion seems possible, since Sho1 and Sh1 are unrelated to each other in primary structure (18, 30). Indeed, they show subtle differences in response to osmotic stress. For example, Sho1 was characterized as activating Hog1 more slowly upon osmotic stress than Sh1 (18). In addition, Sho1 does not respond as well to severe osmotic stress as the two-component system (39). How Sho1 might respond to osmotic stress or heat stress is not known. Perhaps it responds by a mechanism similar to that of other heat stress sensors by undergoing a change in oligomerization. For example, heat stress induces heat shock transcription factor to trimerize into its active form (43), while the Salmonella transcriptional repressor, TlpA, undergoes a heat-induced transition from an active dimer to an inactive monomer (12). How the bacterial chemotaxis receptors or thermosensors sense heat stress is not certain; however, changes in methylation can switch the Tar warmth sensor into a cold sensor (17, 24, 26).

The possible role of heat stress activation of Hog1 was also explored. Hog1 is not essential during heat stress. However, we found that a hog1Δ strain recovered more slowly from heat stress than the wild type (Fig. 5). Although modest, the defect of the hog1Δ strain in competition with the wild type would be a significant disadvantage. How heat stress-activated Hog1 facilitates recovery is not clear. Although osmotic stress activation of Hog1 resulted in its nuclear accumulation, heat stress activation did not significantly alter its localization (data not shown). Therefore, heat stress-activated Hog1 is not likely to alter gene expression but may exert its effect by phosphorylating cytoplasmic proteins.

We also examined the function of PTPs during heat stress and found that they have at least two roles. As described above, one role is to prevent hyperactivation of Hog1. A similar function has been established for PTPs regulating osmotic stress activation of Hog1 and for PTPs controlling other MAPKs in yeast organisms (13, 23, 44, 45). In a ptp2Δ ptp3Δ mutant, heat stress increased Hog1 activity ~6-fold over that in the wild type (Fig. 6A) and osmotic stress increased Hog1 activity 4-fold over that in the wild type (46). One unexplained observation is that heat stress is lethal to the ptp2Δ ptp3Δ strain, while osmotic stress is not (45). The simplest explana-
The means by which PTPs block erroneous cross talk likely involves binding and dephosphorylation of MAPKs. For example, the lack of PTPs would inhibit Hog1-pY dephosphorylation and facilitate access of Mkk1-386 to Hog1, allowing Mkk1-386 to activate Hog1 directly. That PTPs could act as inhibitors by binding Hog1 is likely, as Ptp2 and Ptp3 bind tightly to Hog1 in yeast lysates (13, 23, 44). These binding interactions are significant in vivo, since the nucleus-localized Ptp2 can drive Hog1 from the cytoplasm to the nucleus, while the cytoplasmic Ptp3 can draw Hog1 out of the nucleus to the cytoplasm (22).
tions, as the catalytically inactive PTPs also shift Hog1 subcellular localization to a localization similar to that seen in the wild type (22). Furthermore, we showed here that the catalytically inactive PTPs suppressed the ptpΔΔ mutant temperature-sensitive defect (Fig. 2C), indicating that phosphatase activity is not necessary for blocking Hog1-dependent lethality. Therefore, by binding and sequestering MAPKs, PTPs could act in a manner analogous to that of scaffold proteins and contribute to specificity in MAPK signaling.

Previous work also suggested the importance of MAPK phosphatases in maintaining specificity in signaling pathways. For example, the sevenmaker mutation of the Drosophila rolled gene encoding PTPK suppresses interaction with MAPK phosphatases (4); the analogous mutation of the pheromone response gene encoding MAPK resists interaction with MAPK phosphatase (3). Two protein-tyrosine phosphatases inactivate the osmotic stress response pathway in yeast by targeting the mitogen-activated protein kinase, Hog1. The P_tg_2 mutant protein (11). These studies suggested that blocking the interaction between a PTPK and its phosphatase suppresses lethality of an N-end rule-dependent mutant. Proc. Natl. Acad. Sci. USA 76:91–95.

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