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, Sln1, the second osmosensor in the HOG pathway, was not involved in heat stress activation of Hog1, suggesting that the Sho1 and Sln1 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 NH$_2$-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 hypot-osmotic stress (6, 10, 14). One aspect of stress response 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 NH$_2$-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 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 specific for phosphothreonine 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 specific for phosphothreonine have been found as MAPK regulators.
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, Sln1 and Sho1, osmosensors. The two-component system, Sln1 and Sho1, negatively regulates the MEKKs Ssk2 and Ssk22, while the Sln1 and Sho1 osmosensors. The two-component system, Sln1 and Sho1, negatively regulates the MEKKs Ssk2 and Ssk22, while 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 Pp2Cps1ct1 and Ptc2 or Ptc3 dephosphorylate the phosphotyrosine residue in the activation loop.

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 deletionants, BY47 and BY48 (2), unless otherwise noted. Fus3 was deleted from BY47 to produce CMY13 (MATa thr1::HIS3). The fus1::KANMX4 allele was obtained from p1B225 (a gift from E. Ellen). Skg1 was deleted from BY47 to produce IMY111 by using the skg1::TRP1 allele from plasmid pSK1::TRP1 described below. Sho1 was deleted from BY47 to produce CMY18 by using the shol::LEU2 allele from pSSkhol::LEU2, described below. IMY114 (ppto::LEU2) was produced by transforming BY47 with the ppto::LEU2 allele, described below. IMY108 (ppto::URA3) was produced by transforming BY47 with the ppto::URA3 allele contained in plasmid pMFA11 (gifts from M. Gustin). Strain IMY17b (pto3::HIS3) was produced by deleting JDS2 (8) with the deletion allele from pCM1 (23). Deletion of each of these genes, Fus3, Skg1, Sho1, Ptp2, Ptp3, and Ptp3, was confirmed by Southern analysis and/or PCR. Strains bearing these deletions in combination with others were produced by standard genetic methods.

The ste11::XmMX, ste20::XmMX, and ste50::XmMX deletion alleles, with the appropriate transformation plasmids, were obtained from S. Al-Zarban, and I. M. Ota, submitted for publication (Fig. 1). To uncover new functions of protein phosphatases, we examined the phenotypes 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.

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
sequences of PTP2. This plasmid was digested with EcoRV and ligated with the fragment described above containing LEU2.

To examine GPD1 expression, plasmid YplpGPD1::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 plasmid Yps75 (URA3, integrating vector) (25) to produce an in-frame fusion to lacZ. The GPD1 fragment was produced by PCR with oligonucleotides 5'-GGGATCCGGAGACTGTTGCCTCTTACTCG-3' and 5'-GGAATTCCTCGAAGCAGCACTG-3'.

Immunoblotting. Heat stress activation of Hog1 was examined by using BY248. Thr-phosphorylated Hog1 (Hog1-thr) was detected by using antiphosphothreonine antibody (VP20; ICN) and dually phosphorylated Hog1 (Hog1-ppThr/Thr) was detected by using antibody specific for dually phosphorylated Hog1 (phospho-Thr148/Ser173) antibody (New England BioLabs, Beverly, MA). To examine heat shock, untreated or heat-shocked cells were collected by centrifugation (2 min at 3,000 rpm), washed twice with ice-cold water, and then resuspended in medium lacking uracil and containing either 2% glucose or 4% galactose. Cells were lysed and immunoblotting was performed as described previously (23). Immunoreactivity was visualized by using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Promega).

Hog1 kinase assay. Strains expressing epitope-tagged Hog1 (Hog1-epitope) were grown to exponential phase (~1 A600 unit) in selective medium at 23°C, heat shocked by the addition of an equal volume of medium at 55°C, and further incubated at 39°C. Cells were harvested by centrifugation and lysed, and kinase assays were performed by using [γ-32P]ATP and myelin basic protein (MBP) as described previously (40). Assays were performed a minimum of two times for each strain tested.

\( ^{a} \) All strains were derived from DF5 unless otherwise indicated.

\( ^{b} \) Derived from JDS2.

\[ \text{TABLE 1. Yeast strains}^a \]

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\( ^{a} \) All strains were derived from DF5 unless otherwise indicated.

\( ^{b} \) Derived from JDS2.

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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 a2CO3, 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 p181HOG1K52Mha3) 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 ptp2Δ ptp3Δ (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.

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 (IMY21b) 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 p181HOG1ha3) was compared to that of a ptp2Δ ptp3Δ hog1Δ52M strain (CMY12 expressing the kinase-inactive mutant Hog1K52M from plasmid p181HOG1K52Mha3) at 37 and 30°C on selective medium.
activation of Hog1 and Mpk1 could be responsible for \textit{ppp2Δ ptp3Δ} temperature sensitivity. If so, then deletion of \textit{MPK1} should suppress the \textit{ppp2Δ ptp3Δ} defect. For this test, the \textit{ppp2Δ ptp3Δ mpklΔ} strain was grown at 37°C on osmomoregulated medium, which is supplemented with an osmotic stabilizer, 1 M sorbitol. It was necessary to do so, since \textit{mpklΔ} cells are inviable at 37°C due to a cell wall lysis defect unless grown on such medium (15). Although the \textit{mpklΔ} strain was viable at 37°C on this medium, the \textit{mpklΔ ptp2Δ ptp3Δ} strain was not (data not shown), suggesting that the temperature sensitivity of the \textit{ppp2Δ ptp3Δ} strain was due primarily to heat stress activation of Hog1 and not Mpk1. We also tested whether \textit{BCK1}, encoding the MEKK in the cell wall integrity pathway, could be involved in \textit{ppp2Δ ptp3Δ} temperature sensitivity. However, the \textit{ppp2Δ ptp3Δ bck1Δ} strain was still nonviable at 37°C on osmomoregulated medium (data not shown). It should be pointed out that one limitation of these experiments was that the osmomoregulated medium required to support the \textit{mpklΔ ptp2Δ ptp3Δ} and \textit{bck1Δ ptp2Δ ptp3Δ} strains would result in the activation of Hog1. However, since the deletion of \textit{HOG1} largely suppressed the temperature sensitivity of the \textit{ppp2Δ ptp3Δ} strain, Hog1 is primarily affected by heat stress in the phosphatase mutant.

\textbf{Ptp2 and Ptp3 temperature sensitivity requires active Hog1.}

The results reported above suggested that \textit{ppp2Δ ptp3Δ} temperature sensitivity is a result of Hog1 kinase hyperactivation. To test this notion, wild-type \textit{HOG1} was substituted with catalytically inactive \textit{hog1K52M}. The \textit{ppp2Δ ptp3Δ hog1K52M} strain grew well at 37°C (Fig. 2B), indicating that Hog1 kinase activity was necessary for the \textit{ppp2Δ ptp3Δ} defect. It follows that \textit{ppp2Δ ptp3Δ} temperature sensitivity is due to an inability to inactivate Hog1 by dephosphorylation of Hog1-P. 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 \textit{ppp2Δ ptp3Δ} strain. As expected, the wild-type \textit{Ptp2 PTP2} 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 \textit{ppp2Δ ptp3Δ} mutant temperature sensitivity.

\textbf{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 \textit{ppp2Δ ptp3Δ} temperature sensitivity. Deletion of the MEK-encoding gene, \textit{Pbs2}, suppressed the \textit{ppp2Δ 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, \textit{Pbs2} was substituted with catalytically in-

active \textit{pbs2K389M}. The \textit{ppp2Δ ptp3Δ pbs2K389M} strain was viable at 37°C (Fig. 3B), indicating that Pbs2 kinase activity was necessary for \textit{ppp2Δ 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-Ssk1, 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 \textit{SHO1} were deleted. \textit{SSK1} was deleted rather than \textit{SLN1}, since removal of the latter is lethal (20, 30). Deletion of both \textit{SSK1} and \textit{SHO1} suppressed the temperature sensitivity of the \textit{ppp2Δ 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 \textit{SHO1} and \textit{SSK1} deletions to suppress \textit{ppp2Δ ptp3Δ} temperature sensitivity was examined. Deletion of \textit{SHO1} largely suppressed \textit{ppp2Δ ptp3Δ} temperature sensitivity, while deletion of \textit{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 \textit{STE20}, \textit{STE50}, and \textit{STE11} genes were deleted. Each of the resulting strains, \textit{ste20Δ ptp2Δ ptp3Δ ste50Δ ptp2Δ ptp3Δ}, and \textit{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.

\textbf{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 \textit{ppp2Δ 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 \textit{PBS2} or deletion of both \textit{SSK1} and \textit{SHO1} blocked heat stress-induced Hog1 activity (Fig. 4B). Deletion of \textit{SHO1} greatly inhibited heat stress activation of Hog1, while deletion of \textit{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 \textit{hog1Δ} strains. Deletion of \textit{HOG1} led to one reproducible heat stress defect. The \textit{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.

FIG. 3. Heat stress sensitivity of the PTP null strain requires Pbs2 and the Sho1 branch of the HOG pathway. (A) Deletion of the MEK-encoding gene, PBS2, alleviates ptp2Δ ptp3Δ temperature sensitivity. A wild-type strain (BBY48) and mutant ptp2Δ ptp3Δ (HFY6b) and ptp2Δ ptp3Δ pbs2Δ (AWY1) strains were grown on YPD medium at 37 and 30°C. (B) Pbs2 kinase activity is required for ptp2Δ ptp3Δ temperature sensitivity. The growth of a ptp2Δ ptp3Δ PBS2 strain (AWY1 expressing wild-type PBS2 from plasmid p111PBS2) was compared to that of a ptp2Δ ptp3Δ pbs2K389M strain (AWY1 carrying the kinase-inactive mutant on plasmid p111PBS2K389M) on selective medium at 37 and 30°C. (C) Sho1, but not the two-component system, is required for ptp2Δ ptp3Δ temperature sensitivity. The growth of strains lacking PTPs and the upstream regulators Sho1 and/or Ssk1 was compared. Wild-type and ptp2Δ ptp3Δ strains were as listed in panel A and were compared to ptp2Δ ptp3Δ ssk1Δ sho1Δ (ACB3), ptp2Δ ptp3Δ sho1Δ (ACB1), and ptp2Δ ptp3Δ ssk1Δ sho1Δ (ACB2) strains grown on YPD medium. (D) Ste20, Ste50, and Ste11 in the Sho1 branch of the HOG pathway are necessary for ptp2Δ ptp3Δ temperature sensitivity. Wild-type and ptp2Δ ptp3Δ strains were as listed in panel A and were compared to ptp2Δ ptp3Δ ste20Δ (KKY1), ptp2Δ ptp3Δ ste50Δ (KKY2), and ptp2Δ ptp3Δ ste11Δ (KKY3) strains grown on YPD medium.
Ptp2 and Ptp3 prevent hyperactivation of Hog1 during heat stress. The temperature sensitivity of the ptp2Δ ptp3Δ strain and its suppression by deletion of HOG1 suggested that ptp2Δ ptp3Δ lethality is due to heat stress hyperactivation of Hog1. To examine this idea further, we assayed Hog1 kinase activity in the ptp2Δ ptp3Δ strain. We expected that the ptp2Δ ptp3Δ 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 ptp2Δ ptp3Δ mutant (46). In our strain background, Hog1 kinase activity was elevated ~10-fold in the ptp2Δ ptp3Δ strain compared to the wild type in the absence of heat stress (Fig. 6A). Consistent with this result, the expression of 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 ptp2Δ ptp3Δ strain was viable at 23°C. Upon a shift to 39°C, Hog1 kinase activity increased in the ptp2Δ ptp3Δ mutant, to a level that was nearly sixfold higher than that in heat-treated wild-type cells. Similarly, GPD1 expression was induced to a higher level in the ptp2Δ ptp3Δ strain (Fig. 6B), indicating that Hog1 activity affected downstream components. The ptp2Δ ptp3Δ 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 PP2Cs 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 ptp2Δ ptp3Δ 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 crosstalk 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 crosstalk between these pathways. Such inappropriate crosstalk 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 null 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 Shl1 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 Shl1 (18). In addition, Shl1 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 (41), 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-pT,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 by binding Hog1 is likely, as Ptp2 and Ptp3 bind tightly to Hog1.
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 ptp2Δ ptp3Δ 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 MAPK Resists interaction with MAPK phosphatases (4); the analogous mutation of the pheromone response pathway, FUS3, allowed osmotic stress to activate the Fus3 mutant protein (11). These studies suggested that blocking the interaction between a MAPK and its phosphatase facilitates erroneous cross talk. In mammalian cells, PTPs have been shown to be involved in cross talk between the protein kinase A and MAPK pathways. The PTPs HePTP and PTP-SL are phosphorylated by protein kinase A, and phosphorylation inhibits their ability to bind and inactivate ERK (3, 36). Last, the levels of negative and positive regulators in MAPK pathways are crucial for specificity. For example, inappropriate cross talk between the HOG and pheromone response MAPK pathways occurs when positive regulators of the HOG pathway are deleted (27). Both MAPK-phosphatase binding interactions and the balanced activity of the kinases and phosphatases in these pathways are necessary to promote specificity in MAPK signaling pathways.

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yeast Pbs2 depends on osmostress, the membrane protein Sho1 and Cdc42.


