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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 *hog1*Δ 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 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 Sln1, 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 Sln1-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).

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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 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 (PP2Cs) 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 PP2Cs Ptc1 (40) and Ptc2 and Ptc3 (C. Young, J. Mapes, J. Hanne-

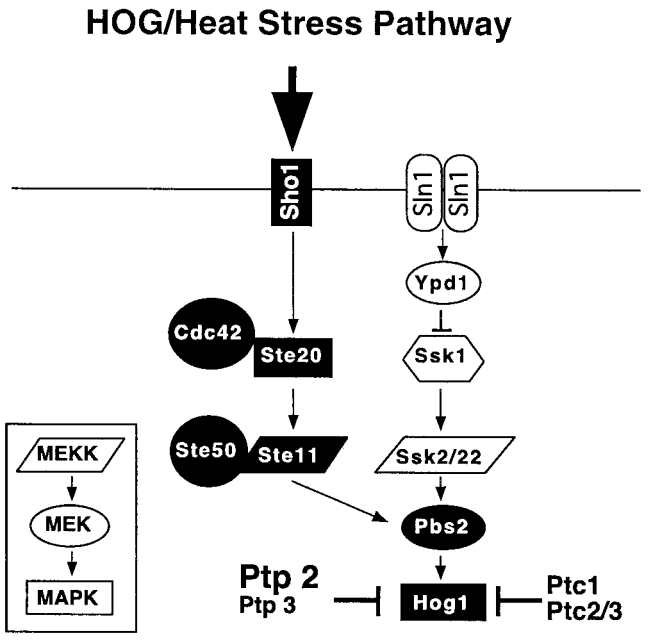


FIG. 1. Osmotic stress versus heat stress activation of the HOG pathway. The HOG pathway is regulated by two membrane-bound proteins, Sln1 and Sho1. Osmotic stress activates the MAPK Hog1 via the Sln1 and Sho1 osmosensors. The two-component system, Sln1-Ypd1-Ssk1, negatively regulates the MEKKs Ssk2 and Ssk22, while Sho1, Ste20, Cdc42, and Ste50 positively regulate the MEKK Ste11. Once the MEKKs 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 phosphothreonine 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 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.

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 dissectants, BBY45 and BBY48 (2), unless otherwise noted. *FUS3* was deleted from BBY48 to produce CMY13 (*MAT*α *fus3-7*::*HIS3*). The *fus3-7*::*HIS3* allele was obtained from pJB225 (a gift from E. Elion). *SSK1* was deleted from BBY45 to produce IMY111 by using the *ssk1*Δ::*TRP1* allele from plasmid pssk1ΔTRP1, described below. *SHO1* was deleted from BBY48 to produce CMY18 by using the *sho1*Δ::*LEU2* allele from pSKsho1ΔLEU2, described below. IMY114 (*ptp2*Δ::*LEU2*) was produced by transforming BBY48 with the *ptp2*Δ::*LEU2* allele, described below. IMY108 (*pbs2*Δ::*URA3*) was produced by transforming BBY48 with the *pbs2*Δ::*URA3* allele contained in plasmid pJB4D, and AWY3 was produced by transforming BBY45 with the *pbs2*Δ::*LEU2* allele contained in plasmid pJMA11 (gifts from M. Gustin). Strain IMY71b (*ptp3*Δ::*HIS3*) was produced by transforming JD52 (8) with the deletion allele from pCM1 (23). Deletion of each of these genes, *FUS3*, *SSK1*, *SHO1*, *PTP2*, *PBS2*, and *PTP3*, was confirmed by Southern analysis or by PCR. Strains bearing these deletions in combination with others were produced by standard genetic methods.

The *ste11*Δ::*kanMX*, *ste20*Δ::*kanMX*, and *ste50*Δ::*kanMX* deletion alleles, which were transformed into *ptp2*Δ *ptp3*Δ strain HFY6b, were produced by PCR with template p400 (5) and appropriate oligonucleotides. AWY5 and AWY6 were produced by transforming AWY4 (*ptp2*Δ::*LEU2 ptp3*::*TRP1 pbs2*Δ::*LEU2*) and ACB3 (*ptp2*Δ::*LEU2 ptp3*Δ::*TRP1 sho1*Δ::*LEU2 ssk1*Δ::*TRP1*), respectively, with the *hog1*Δ::*KanNMX* allele, which was produced by PCR with appropriate oligonucleotides. Deletion of each of these genes, *STE11*, *STE20*, *STE50*, and *HOG1*, was confirmed by PCR.

To examine the role of PTP catalytic activity in *ptp2*Δ *ptp3*Δ temperature sensitivity, *ptp2*Δ *ptp3*Δ strains carrying wild-type PTPs, catalytically inactive PTPs, and empty vectors were produced. JHY1 (*ptp2*Δ::*HIS3 ptp3*Δ::*HIS3*) was transformed with multicopy plasmids expressing wild-type PTPs, p112PTP2 (*TRP1*, 2μm) and p181PTP3 (*LEU2*, 2μm) (13); mutant PTPs, p112PTP2C666S (*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 *ptp2*Δ *ptp3*Δ strains were constructed that expressed β-galactosidase under the regulation of the *GPD1* promoter. A yeast integrating plasmid, YIpGPD1::lacZ, was produced (see below), digested at a unique *Nar*I site 353 bp upstream of the *GPD1* start codon, and transformed into DF5 (2). Ura^+ transformants were selected, and Southern analysis was performed to confirm integration at the *GPD1* locus. Heterozygous diploids bearing the *GPD1*::*lacZ* fusion were sporulated and dissected to produce haploid strain CMY15 (*MAT*α *GPD1*::*lacZ*::*URA3*). To produce a *ptp2*Δ *ptp3*Δ strain expressing *GPD1*::*lacZ*, CMY15 and HFY6 (*MAT***a** *ptp2*Δ::*HIS3 ptp3*Δ::*TRP1*) were mated, diploids were sporulated, and tetrads were dissected. Ura^+ His^+ Trp^+ spore clones were isolated as *ptp2*Δ::*HIS3 ptp3*Δ::*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 *Not*I restriction site by PCR, and an ~100-bp *Not*I fragment containing three repeats of the ha epitope (ha3) was ligated. A 1.5-kb *Sal*I-*Kpn*I fragment containing the 3′ end of *HOG1* fused to ha3 was ligated, together with a 1.1-kb *Sal*I 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 *hog1*Δ strain. The *hog1K52M* allele was produced by PCR with the mutagenic oligonucleotide 5′-CATCTCAGCCAGTTGCCATTATG AAAATCATG-3′ (the mutations are underlined). A 500-bp *Eco*RI fragment containing the mutation was substituted for the wild-type *Eco*RI fragment in p181HOG1ha3 to produce p181HOG1K52Mha3. This plasmid did not complement the osmotic stress sensitivity of a *hog1*Δ strain.

Plasmids expressing wild-type *PBS2* (p111PBS2) and catalytically inactive *pbs2K389M* (p111PBS2K389M) were also produced. *PBS2*, contained in an ~3.4-kb genomic *Cla*I-*Sac*I fragment, was ligated to the multicopy vector pRS423 (*HIS3*, 2μm) to produce p423PBS2. The ~3.4-kb *Sal*I-*Sac*I fragment from p423PBS2 was cloned into the low-copy-number vector YCplac111 (*LEU2*, CEN/ARS) (9) to produce p111PBS2. The *pbs2K389M* mutation was introduced by using a QuikChange site-directed mutagenesis kit (Stratagene). A 707-bp *Eco*RV fragment containing the K389M mutation was substituted for the wild-type fragment in p111PBS2 to produce p111PBS2K389M.

To delete *SSK1*, *SHO1*, and *PTP2*, the following plasmids were constructed. A plasmid containing the 5′- and 3′-flanking sequences of *SSK1* (pUCssk1) was produced by PCR, and the *TRP1* gene was inserted to produce pssk1ΔTRP1. To delete *SHO1*, a plasmid containing its 5′- and 3′-flanking sequences (pSKsho1) was constructed by PCR, and the *LEU2* gene, contained in a 2.2-kb *Sal*I-*Xho*I fragment, was inserted to produce pSKsho1ΔLEU2. The *ptp2*Δ::*LEU2* allele was constructed by using plasmid pHS4.85 (29), which contains 5′- and 3′-flanking

TABLE 1. Yeast strains[a]

Strain	Genotype	Reference or source
DF5	MAT**a**/MATα *trp1-1/trp1-1 ura3-52/ura3-52 his3-Δ200/his3-Δ200 leu2-3, 112/leu2-3, 112/lys2-801/lys2-801 gal/gal*	Bartel et al. (2)
BBY45	*MAT***a** *trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	Bartel et al. (2)
BBY48	*MAT*α *trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	Bartel et al. (2)
IMY21b	*MAT*α *ptp2Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	Ota and Varshavsky (30)
HFY2	*MAT*α *ptp3Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	Jacoby et al. (13)
HFY6b	*MAT*α *ptp2Δ::HIS3 ptp3Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	Jacoby et al. (13)
CMY10b	*MAT*α *ptp2Δ::HIS3 hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	Mattison and Ota (22)
CMY12	*MAT*α *ptp2Δ::HIS3 ptp3Δ::TRP1 hog 1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	Mattison and Ota (22)
CMY13	*MAT*α *fus3-7::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
CMY14	*MAT*α *ptp2Δ::HIS3 ptp3Δ::TRP1 fus3-7::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
CMY8	*MAT*α *ptp2Δ::HIS3 ptp3Δ::TRP1 mpk1Δ::HIS3 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	Mattison et al. (23)
JHY1	*MAT*α *ptp2Δ::HIS3 ptp3Δ::HIS3 trp1-Δ::63 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 GAL*$^+$	This study
IMY108	*MAT*α *pbs2Δ::URA3 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
AWY1	*MAT*α *ptp2Δ::HIS3 ptp3Δ::TRP1 pbs2Δ::URA3 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
IMY111	*MAT***a** *ssk1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
CMY18	*MAT*α *sho1Δ::LEU2 trp1-1 ura3-52 his 3-Δ200 leu2-3, 112 lys2-801 gal*	This study
IMY114	*MAT*α *ptp2Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
ACB1	*MAT***a** *ptp2Δ::LEU2 ptp3Δ::TRP1 sho1Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
ACB2	*MAT***a** *ptp2Δ::LEU2 ptp3Δ::TRP1 ssk1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
ACB3	*MAT*α *ptp2Δ::LEU2 ptp3Δ::TRP1 sho1Δ::LEU2 ssk1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
KKY1	*MAT*α *ste20Δ::kanMX ptp2Δ::HIS3 ptp3Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
KKY2	*MAT*α *ste50Δ::kanMX ptp2Δ::HIS3 ptp3Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
KKY3	*MAT*α *ste11Δ::kanMX ptp2Δ::HIS3 ptp3Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
IMY100	*MAT*α *hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	Jacoby et al. (13)
SGY2	*MAT*α *pbs2Δ::URA3 hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
CAY1	*MAT*α *sho1Δ::LEU2 ssk1Δ::TRP1 hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
CAY8	*MAT*α *ssk1Δ::TRP1 hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
CAY3	*MAT*α *sho1Δ::LEU2 hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
CAY6	*MAT*α *ptp2Δ::LEU2 ptp3Δ::TRP1 hog1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	This study
CMY15	*MAT*α *GPD1::lacZ::URA3 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
CMY16	*MAT*α *ptp2Δ::HIS3 ptp3Δ::TRP1 GPD1::lacZ::URA3 trp1-1 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 gal*	This study
AWY3	*MAT***a** *pbs2Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	This study
AWY4	*MAT***a** *ptp2Δ::LEU2 ptp3::TRP1 pbs2Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	This study
AWY5	*MAT***a** *ptp2Δ::LEU2 ptp3Δ::TRP1 hog1Δ::kanMX pbs2Δ::LEU2 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	This study
AWY6	*MAT***a** *ptp2Δ::HIS3 ptp3Δ::TRP1 hog1Δ::kanMX sho1Δ::LEU2 ssk1Δ::TRP1 trp1-1 ura3-52 his3-Δ200 leu2-3,112 lys2-801 gal*	This study
JD52	*MAT***a** *trp1-Δ63 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 GAL*$^+$	Ghislain et al. (8)
IMY71b[b]	*MAT***a** *ptp3Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 GAL*$^+$	This study
CMY23[b]	*MAT***a** *ptp2Δ::HIS3 ptp3Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3, 112 lys2-801 GAL*$^+$	Mattison et al. (23)
CMY24[b]	*MAT***a** *ptp2Δ::HIS3 ptp3Δ::HIS3 hog1Δ::TRP1 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 GAL*$^+$	This study

[a] All strains were derived from DF5 unless otherwise indicated.
[b] Derived from JDS2.

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

To examine *GPD1* expression, plasmid YIpGPD1::lacZ, a yeast integrating vector containing the *GPD1* promoter fused to the *lacZ* gene (*GPD1::lacZ*), was constructed. An 813-bp *Bam*HI-*Sal*I *GPD1* fragment, containing 462 bp upstream of the start codon and 351 bp downstream of the start codon, was ligated to plasmid YIp357 (*URA3*, integrating vector) (25) to produce an in-frame fusion to *lacZ*. The *GPD1* fragment was produced by PCR with oligonucleotides 5′-GGGATCCGAGACTGTTGTCCTCCTACTG-3′ and 5′-GGAATTCCCAGATGCTAGAAGCAACTGTG-3′.

Immunoblotting. Heat stress activation of Hog1 was examined by using BBY48. Tyr-phosphorylated Hog1 (Hog1-pY) was detected by using antiphosphotyrosine antibody (PY20; ICN), and dually phosphorylated Hog1 was detected by using antibody specific for dually phosphorylated Hog1 (phospho-p38 antibody; New England BioLabs) (40). To examine Hog1 activation in *MKK1-386*-expressing cells, exponential cultures of JD52 (*MAT***a** *trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 GAL*$^+$) and an isogenic *ptp2Δ ptp3Δ* strain, CMY23 (*MAT***a** *ptp2Δ::HIS3 ptp3Δ::HIS3 trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801 GAL*$^+$), each carrying plasmid pNV7MKK1-386 (41), were grown 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-ha) were grown to exponential phase (~1 A_{600} 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-^{32}P]ATP and myelin basic protein (MBP) as described previously (40). Assays were performed a minimum of two times for each strain tested.

***o*-Nitrophenyl-β-D-galactopyranoside assay.** *GPD1* expression was examined with the wild type (CMY15) and the *ptp2Δ ptp3Δ* mutant (CMY16) expressing *GPD1::lacZ*. Cultures were grown in yeast extract-peptone-dextrose (YPD) to ~1 A_{600} unit and were left untreated or heat shocked by the addition of an equal volume of YPD at 55°C. Cells were further incubated at 39°C, and 10-ml aliquots were pelleted by centrifugation at various times. Cells were resuspended in 600 μl of Z buffer (100 mM $NaPO_4$ [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, 38.6 mM β-mercaptoethanol), and to 500 μl of the cell suspension, 30 μl of chloroform

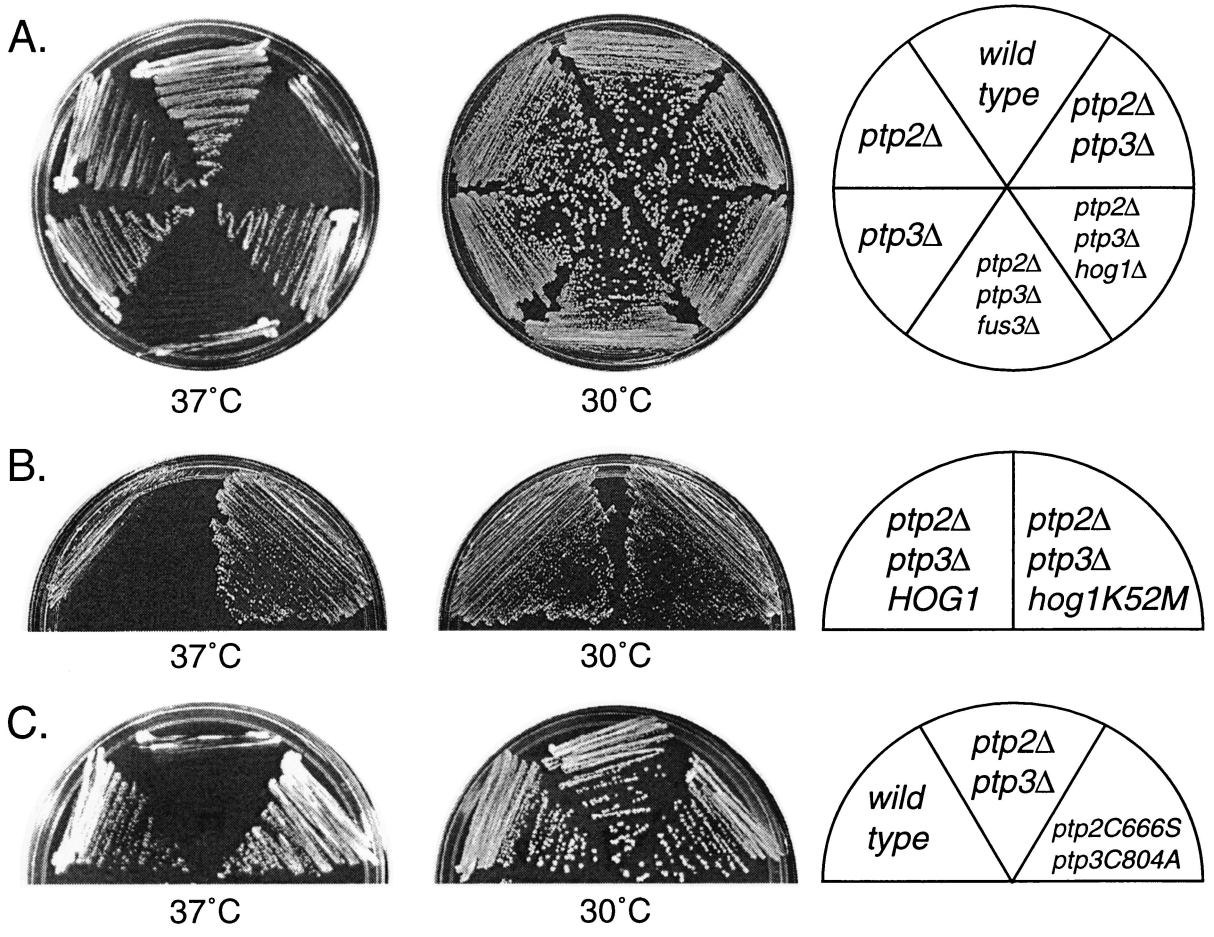


FIG. 2. Strains lacking the PTPs Ptp2 and Ptp3 are temperature sensitive due to Hog1 hyperactivation. (A) Growth of the wild type and strains lacking PTPs, either alone or in combination with a *HOG1* or a *FUS3* deletion, was examined under heat stress. A wild-type strain (BBY48) and isogenic *ptp2*Δ *ptp3*Δ (HFY6b), *ptp2*Δ *ptp3*Δ *hog1*Δ (CMY12), *ptp2*Δ *ptp3*Δ *fus3*Δ (CMY14), *ptp3*Δ (HFY2), and *ptp2*Δ (IMY21b) strains were grown on standard rich medium, YPD, at 37 or 30°C for 3 days. (B) Hog1 kinase activity is necessary for *ptp2*Δ *ptp3*Δ temperature sensitivity. The growth of a *ptp2*Δ *ptp3*Δ *HOG1* strain (CMY12 *ptp2*Δ *ptp3*Δ *hog1*Δ carrying plasmid p181HOG1ha3) was compared to that of a *ptp2*Δ *ptp3*Δ *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 *ptp2*Δ *ptp3*Δ temperature sensitivity. The wild type (JHY1 *ptp2*Δ *ptp3*Δ expressing wild-type PTPs on multicopy plasmids p112PTP2 and p181PTP3), mutant *ptp2*Δ *ptp3*Δ (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.

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 Na_2CO_3, and the A_{420} 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 MAPK signaling (13, 19). While examining the phenotype of strains lacking PTPs, we found that a *ptp2*Δ *ptp3*Δ double mutant was unable to grow at 37°C, although it was viable at 30°C (Fig. 2A). A *ptp2*Δ single mutant showed a slight defect at 37°C, while a *ptp3*Δ strain showed no defect (Fig. 2A); neither strain showed a defect at 30°C. The temperature-sensitive phenotype of the *ptp2*Δ *ptp3*Δ and *ptp2*Δ 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 *ptp2*Δ *ptp3*Δ and *ptp2*Δ 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 *ptp2*Δ *ptp3*Δ *hog1*Δ triple mutant grew nearly as well as the wild type at 37°C (Fig. 2A), and a *ptp2*Δ *hog1*Δ strain showed no defect (data not shown), suggesting that Hog1 activation is largely responsible for the temperature sensitivity of the *ptp2*Δ *ptp3*Δ strain. In support of this conclusion, deletion of *FUS3*, encoding the MAPK in the pheromone response pathway, did not suppress *ptp2*Δ *ptp3*Δ temperature sensitivity (Fig. 2A).

Ptp2 and Ptp3 also regulate the MAPK Mpk1 in the cell wall integrity pathway (23), and Mpk1 is activated by heat stress (14). Therefore, it seemed possible that simultaneous hyper-

activation of Hog1 and Mpk1 could be responsible for *ptp2*Δ *ptp3*Δ temperature sensitivity. If so, then deletion of *MPK1* should suppress the *ptp2*Δ *ptp3*Δ defect. For this test, the *ptp2*Δ *ptp3*Δ *mpk1*Δ 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 *mpk1*Δ cells are inviable at 37°C due to a cell wall lysis defect unless grown on such medium (15). Although the *mpk1*Δ strain was viable at 37°C on this medium, the *mpk1*Δ *ptp2*Δ *ptp3*Δ strain was not (data not shown), suggesting that the temperature sensitivity of the *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ temperature sensitivity. However, the *ptp2*Δ *ptp3*Δ *bck1*Δ 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 *mpk1*Δ *ptp2*Δ *ptp3*Δ and *bck1*Δ *ptp2*Δ *ptp3*Δ strains would result in the activation of Hog1. However, since the deletion of *HOG1* largely suppressed the temperature sensitivity of the *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ temperature sensitivity is a result of Hog1 kinase hyperactivation. To test this notion, wild-type *HOG1* was substituted with catalytically inactive *hog1K52M*. The *ptp2*Δ *ptp3*Δ *hog1K52M* strain grew well at 37°C (Fig. 2B), indicating that Hog1 kinase activity was necessary for the *ptp2*Δ *ptp3*Δ defect. It follows that *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ temperature sensitivity. Deletion of the MEK-encoding gene, *PBS2*, suppressed the *ptp2*Δ *ptp3*Δ 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 inactive *pbs2K389M*. The *ptp2*Δ *ptp3*Δ *pbs2K389M* strain was viable at 37°C (Fig. 3B), indicating that Pbs2 kinase activity was necessary for *ptp2*Δ *ptp3*Δ 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 *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 *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ temperature sensitivity was examined. Deletion of *SHO1* largely suppressed *ptp2*Δ *ptp3*Δ 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, *ste20*Δ *ptp2*Δ *ptp3*Δ, *ste50*Δ *ptp2*Δ *ptp3*Δ, and *ste11*Δ *ptp2*Δ *ptp3*Δ, 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 *ptp2*Δ *ptp3*Δ 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 *hog1*Δ strains. Deletion of *HOG1* led to one reproducible heat stress defect. The *hog1*Δ strain recovered more slowly from heat stress than wild type (Fig. 5). Both mutant and wild-type strains were grown at 23°C,

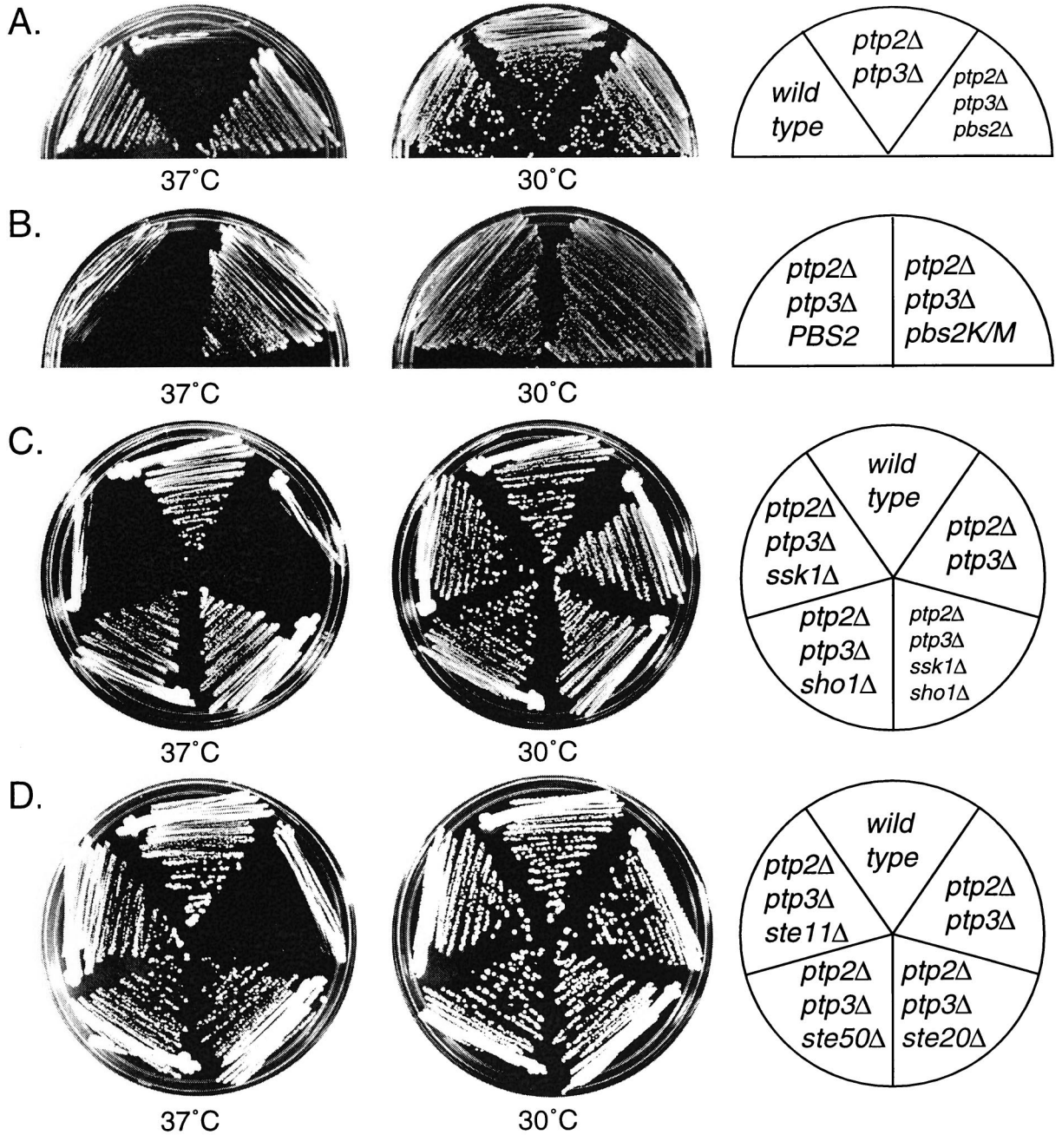
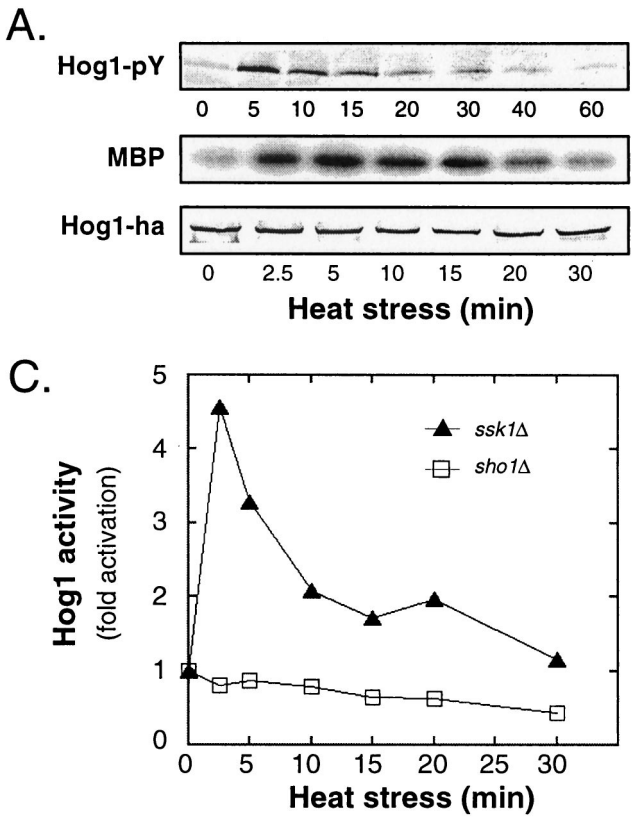


FIG. 3. Heat stress sensitivity of the PTP null strain requires Pbs2 and the Sho1 branch of the HOG pathway. (A) Deletion of the MEK-encoding gene, *PBS2*, alleviates *ptp2*Δ *ptp3*Δ temperature sensitivity. A wild-type strain (BBY48) and mutant *ptp2*Δ *ptp3*Δ (HFY6b) and *ptp2*Δ *ptp3*Δ *pbs2*Δ (AWY1) strains were grown on YPD medium at 37 and 30°C. (B) Pbs2 kinase activity is required for *ptp2*Δ *ptp3*Δ temperature sensitivity. The growth of a *ptp2*Δ *ptp3*Δ *PBS2* strain (AWY1 expressing wild-type *PBS2* from plasmid p111PBS2) was compared to that of a *ptp2*Δ *ptp3*Δ *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 *ptp2*Δ *ptp3*Δ temperature sensitivity. The growth of strains lacking PTPs and the upstream regulators Sho1 and/or Ssk1 was compared. Wild-type and *ptp2*Δ *ptp3*Δ strains were as listed in panel A and were compared to *ptp2*Δ *ptp3*Δ *ssk1*Δ *sho1*Δ (ACB3), *ptp2*Δ *ptp3*Δ *sho1*Δ (ACB1), and *ptp2*Δ *ptp3*Δ *ssk1*Δ (ACB2) strains grown on YPD medium. (D) Ste20, Ste50, and Ste11 in the Sho1 branch of the HOG pathway are necessary for *ptp2*Δ *ptp3*Δ temperature sensitivity. Wild-type and *ptp2*Δ *ptp3*Δ strains were as listed in panel A and were compared to *ptp2*Δ *ptp3*Δ *ste20*Δ (KKY1), *ptp2*Δ *ptp3*Δ *ste50*Δ (KKY2), and *ptp2*Δ *ptp3*Δ *ste11*Δ (KKY3) strains grown on YPD medium.

shifted to 39°C for 22 h, and allowed to recover at 23°C. The *hog1*Δ strain showed a reproducible growth lag relative to wild type after this treatment (Fig. 5). After an additional 10 h of recovery, the *hog1*Δ colonies were similar in size and number to wild type (data not shown). Therefore, the *hog1*Δ strain shows delayed growth in response to heat stress from which it is able to recover, suggesting Hog1 can facilitate recovery from heat stress.



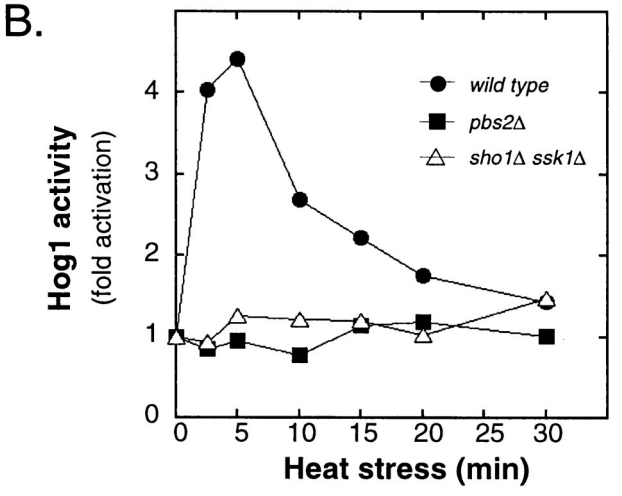


FIG. 4. Hog1 is activated by heat stress in a Pbs2- and Sho1-dependent manner. (A) Hog1 is activated in a strain expressing wild-type PTPs. Hog1-pY was examined in strain IMY100 (*hog1*Δ::*TRP1*) carrying plasmid p181HOG1, where *HOG1* is expressed from the multicopy plasmid YEplac181. The cells were grown 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. Hog1-pY was monitored by immunoblotting with an antiphosphotyrosine antibody (PY20) as described previously (23). Hog1 kinase activity (MBP phosphorylation) increased in response to heat stress. A *hog1*Δ strain (IMY100) expressing Hog1-ha from plasmid pHOG1-ha2 (40) was grown at 23°C or heat stressed at 39°C for the indicated times. Hog1-ha was immunoprecipitated and incubated with the MAPK substrate MBP and [γ-^{32}P]ATP. ^{32}P incorporation into MBP was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and PhosphorImager analysis (Molecular Dynamics). The level of Hog1-ha was constant throughout the time course, as shown by immunoblotting with anti-ha antibody (12CA5; Babco). Thus, heat stress increased Hog1 activity and not the level of Hog1 protein. (B) Heat stress activation of Hog1 is dependent on Pbs2 and the upstream regulators Sho1 and/or Ssk1. Heat stress activation of Hog1 was examined in the following strains expressing Hog1-ha from plasmid pHOG1-ha2: wild type (IMY100 *hog1*Δ), *pbs2*Δ (SGY2 *pbs2*Δ *hog1*), and *sho1*Δ *ssk1*Δ (CAY1 *sho1*Δ *ssk1*Δ *hog1*Δ). Kinase assays were performed and quantified as described for panel A. Immunoblotting showed that the level of Hog1 was constant throughout the time course (data not shown). (C) Heat stress activation of Hog1 is dependent on Sho1, but not the two-component signal transduction pathway. Hog1 kinase activity was compared in a *sho1*Δ strain (CAY3 *sho1*Δ *hog1*Δ carrying pHOG1-ha2) and an *ssk1*Δ strain (CAY8 *ssk1*Δ *hog1*Δ carrying pHOG1-ha2) as described above. Quantitation was done as described for panel A. Immunoblotting showed no difference in the level of Hog1-ha (data not shown).

Ptp2 and Ptp3 prevent hyperactivation of Hog1 during heat stress. The temperature sensitivity of the *ptp2*Δ *ptp3*Δ strain and its suppression by deletion of *HOG1* suggested that *ptp2*Δ *ptp3*Δ lethality is due to heat stress hyperactivation of Hog1. To examine this idea further, we assayed Hog1 kinase activity in the *ptp2*Δ *ptp3*Δ strain. We expected that the *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ mutant (46). In our strain background, Hog1 kinase activity was elevated ~10-fold in the *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ strain was viable at 23°C. Upon a shift to 39°C, Hog1 kinase activity increased in the *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ strain (Fig. 6B), indicating that Hog1 activity affected downstream components. The *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ strain but not in the wild-type *PTP* strain (Fig. 6C). Furthermore, we found that *MKK1-386* was lethal for the *pbs2*Δ *ptp2*Δ *ptp3*Δ 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.

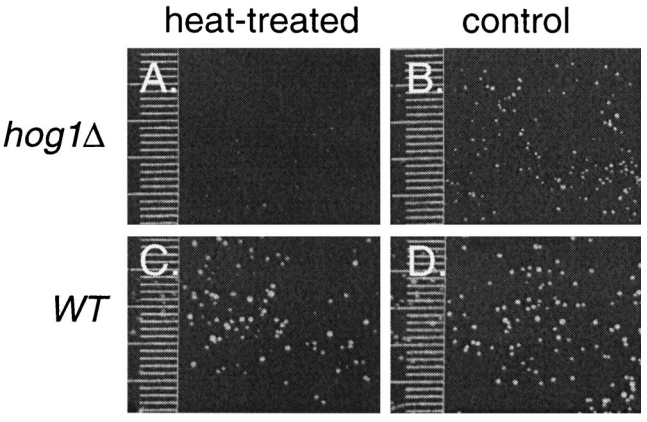


FIG. 5. Hog1 is required for rapid recovery from heat stress. A wild-type (*WT*) strain (BBY45) and an isogenic *hog1*Δ strain were grown to early log phase in liquid YPD medium at 23°C and then plated on solid YPD medium. Plates A and C were heat treated at 39°C for 22 h and allowed to recover at 23°C for 38 h. The untreated controls, plates B and D, were incubated at 23°C for 35 h.

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 *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ 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 Sln1 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 Sln1 (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 *hog1*Δ strain recovered more slowly from heat stress than the wild type (Fig. 5). Although modest, the defect of the *hog1*Δ 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 *ptp2*Δ *ptp3*Δ 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 *ptp2*Δ *ptp3*Δ strain, while osmotic stress is not (45). The simplest explana-

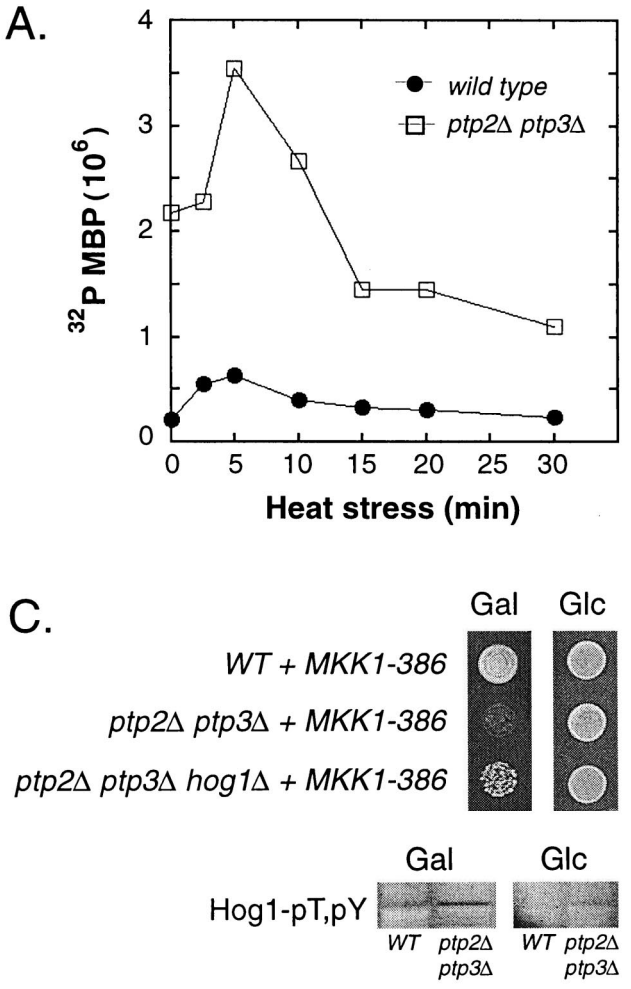


FIG. 6. Ptp2 and Ptp3 prevent heat stress hyperactivation of Hog1 and cross talk with the cell wall integrity MAPK pathway. (A) Hog1 is hyperactivated in a *ptp2*Δ *ptp3*Δ strain. Hog1 kinase activity was compared in a wild-type strain (IMY100 *hog1*Δ carrying pHOG1-ha2) and a *ptp2*Δ *ptp3*Δ strain (CAY6 *ptp2*Δ *ptp3*Δ *hog1*Δ carrying pHOG1-ha2) at 23°C and after a shift to 39°C for the indicated times. Kinase assays were performed as described in the legend to Fig. 4. Quantitation was done by PhosphorImager analysis. Immunoblotting showed that the level of Hog1-ha did not vary during the time course in each strain (data not shown). (B) *GPD1* expression is elevated in a *ptp2*Δ *ptp3*Δ strain. A *GPD1*::*lacZ* reporter was used to examine *GPD1* expression in a wild-type strain (CMY15) and a *ptp2*Δ *ptp3*Δ strain (CMY16). Cells were grown at 23°C and shifted to 39°C for the indicated times. *GPD1* expression was monitored by assaying β-galactosidase (β-gal) activity with *o*-nitrophenyl-β-D-galactopyranoside as a substrate. The average of four independent experiments normalized to cell density is shown plotted against time. Error bars indicate standard deviations. (C) The cell wall integrity pathway activates the HOG pathway when PTPs are deleted. Overexpression of the cell wall integrity pathway hyperactive MEK allele *MKK1-386* is lethal in a *ptp2*Δ *ptp3*Δ strain and is alleviated by deletion of *HOG1*. *MKK1-386* was expressed from the *GAL* promoter by use of plasmid pNV7MKK1-386 (41) in a *ptp2*Δ *ptp3*Δ strain (CMY23) and an isogenic *ptp2*Δ *ptp3*Δ *hog1*Δ strain (CMY24). Growth was compared on galactose-containing medium on which *MKK1-386* was overexpressed and on glucose-containing medium on which it was repressed. Overexpression of *MKK1-386* activated Hog1 in the *ptp2*Δ *ptp3*Δ strain but not in the wild type (*WT*). Hog1 activation was examined in the *ptp2*Δ *ptp3*Δ strain (CMY23) and its wild-type parent, JD52, where both strains carried pNV7MKK1-386. Lysates were prepared from cells prior to and following induction of *MKK1-386* expression on galactose. Immunoblotting was performed by using antibody specific for dually phosphorylated Hog1 (Hog1-pT,pY). A control experiment with a *hog1*Δ strain showed that the band detected by immunoblotting was Hog1 (data not shown).

tion is that after heat stress, the activity of Hog1 in the *ptp2*Δ *ptp3*Δ strain crosses a threshold which, when combined with other physiological changes induced by heat stress, is lethal.

Another potential role of PTPs is to prevent inappropriate cross talk between MAPK pathways. Such a role for MAPK phosphatases might be expected, as Ptp2 and Ptp3 regulate multiple MAPKs, including Hog1, Mpk1, and Fus3 (13, 23, 44, 45). Here, we showed that deletion of *PTP2* and *PTP3* facilitates inappropriate cross talk between the HOG and cell wall integrity pathways. *MKK1-386*, a hyperactive MEK allele in the cell wall pathway, produced *HOG1*-dependent lethality only when PTPs were deleted (Fig. 6C). Furthermore, induction of *MKK1-386* activated Hog1 when PTPs were absent but not when PTPs were present (Fig. 6C). We also found that although deletion of the upstream regulators *SHO1* and *SSK1* or of *SHO1* alone led to a complete failure of heat stress to activate Hog1 when PTPs were present (Fig. 4B and C), a slight increase in Hog1 activity upon heat stress could be detected when PTPs were absent (data not shown). We believe that deletion of PTPs leaves Hog1 unprotected in such a way that other stress-activated pathways, such as the cell wall integrity pathway, can activate it. Taken together, these results suggest that the removal of PTPs leads to the activation of Hog1 by pathways that do not normally act on it.

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). We have shown that these effects are due to binding interac-

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 *ptp2*Δ *ptp3*Δ 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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