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Regulation of the *Saccharomyces cerevisiae* Slt2 Kinase Pathway by the Stress-inducible Sdp1 Dual Specificity Phosphatase*

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The Slt2/Mpk1 mitogen-activated protein kinase (MAPK) cell integrity pathway is involved in maintenance of cell shape and integrity during vegetative growth and mating in *Saccharomyces cerevisiae*. Slt2 is activated by dual phosphorylation of a threonine and tyrosine residue in response to several environmental stresses that perturb cell integrity. Negative regulation of Slt2 is achieved via dephosphorylation by two protein-tyrosine phosphatases, Ptp2 and Ptp3, and a dual specificity phosphatase, Msg5. In this study, we provide genetic and biochemical evidence that the stress-inducible dual specificity phosphatase, Sdp1, negatively regulates Slt2 by direct dephosphorylation. Deletion of *SDP1* exacerbated growth defects due to overexpression of Mkk1^{p386}, a constitutively active mutant of Slt2 MAPK kinase, whereas overexpression of Sdp1 suppressed lethality caused by Mkk1^{p386} overexpression. The heat shock-induced phosphorylation level of Slt2 was elevated in an *sdp1*Δ strain compared with that of the wild type, and heat shock-activated phospho-Slt2 was dephosphorylated by recombinant Sdp1 *in vitro*. Under normal growth conditions, an Sdp1-GFP fusion protein was localized to both the nucleus and cytoplasm. However, the Sdp1-GFP protein translocated to punctate spots throughout the cell after heat shock. *SDP1* transcription was induced by several stress conditions in an Msn2/4-dependent manner but independent of the Rlm1 transcription factor, a downstream target activated by Slt2. Induction of *SLT2* by high osmolarity was dependent on Rlm1 transcription factor and Hog1 kinase, suggesting cross-talk between Slt2 and Hog1 MAPK pathways. These studies demonstrate regulation of Slt2 activity and gene expression in coordination with other stress signaling pathways.

Mitogen-activated protein kinase (MAPK)[1] pathways are evolutionarily conserved signal transduction cascades connecting extracellular stimuli to a wide range of cellular responses. The MAPK cascades are sequential phosphorylation-mediated activation of three kinases, MAPK kinase kinase, MAPK kinase, and MAPK (1, 2). Activation of MAPK requires phosphorylation of both threonine and tyrosine residues of a TXY motif in the activation loop. Therefore, inactivation of MAPK can be achieved by dephosphorylation of either of the two phosphorylation sites. It has been demonstrated that three types of phosphatases, protein-tyrosine phosphatase, serine/threonine phosphatase, and dual specificity phosphatase, are involved in negative regulation of MAPK from yeast to mammals (3).

Saccharomyces cerevisiae encodes five MAPKs involved in distinct cellular responses (4). The four MAPKs present in vegetative cells, Fus3, Kss1, Hog1, and Slt2/Mpk1, are involved in the mating-pheromone response, filamentation-invasion pathway, high osmolarity growth, and cell integrity pathway, respectively. It has been known that two protein-tyrosine phosphatases, Ptp2 and Ptp3, a dual specificity phosphatase Msg5, and type 2C serine/threonine phosphatases are involved in differential inactivation of distinct MAPKs. Ptp3 dephosphorylates Fus3 to maintain a low basal activity and to inactivate Fus3 following pheromone stimulation, and its homologue Ptp2 also plays a minor role as a Fus3 phosphatase (5). Ptp2 and Ptp3 are also involved in negative regulation of Hog1 and Slt2 for maintaining low basal activities and for adaptation following osmotic stress and heat shock, respectively (6–9). However, for Hog1 and Slt2 kinases, Ptp2 is more effective than Ptp3. Targets of the Msg5 dual specificity phosphatase include Fus3, Slt2, and Kss1 (5, 10–12). Msg5 is involved in recovery from pheromone-induced G_1 arrest by dephosphorylating Fus3 kinase (11). The type 2C protein phosphatase Ptc1 inactivates Hog1 for maintaining low basal levels of Hog1 activity and adaptation in response to osmotic stress (13). A genetic interaction suggests an involvement of Ptc1 in the protein kinase C pathway; however, there is currently no evidence for direct dephosphorylation of Slt2 by Ptc1 (14).

The Slt2 cell integrity pathway is involved in maintenance of cell shape and integrity during vegetative growth and mating. This pathway is activated by several environmental stimuli such as heat shock (15), hypoosmotic stress (16), mating pheromone (17), agents causing cell wall stress (18), and actin depolymerization (19). Putative sensors of the Slt2 pathway are the transmembrane proteins Wsc1 (20) and Mid2 (21), which interact with the GDP/GTP exchange factor Rom2 to stimulate GTP loading of the small GTP-binding protein Rho1 (22). Rho1 binds and activates Pkc1, which elicits serial activation of the Slt2 MAPK module composed of MAPK kinase kinase (Bck1), two redundant MAPK kinases (Mkk1/Mkk2), and a MAPK (Slt2) (23). The Rlm1 (24) and SBF (25, 26) transcription factors are two downstream targets of Slt2. Most Rlm1-regulated genes encode cell wall proteins or enzymes involved in cell wall biosynthesis (27). SBF is a heterodimer complex composed of the Swi4 and Swi6 proteins, which regulates gene expression during the G_1/S transition of the cell cycle (28). SBF-activated genes are involved in budding and in membrane and cell wall biosynthesis (29). It has been shown that Slt2 is down-regu-

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; SC, synthetic complete; GFP, green fluorescent protein; GAD, Gal4 activation domain; GBD, Gal4 DNA binding domain; HA, hemagglutinin; PKA, protein kinase A; GST, glutathione *S*-transferase.

lated by the phosphatases Ptp2, Ptp3, and Msg5. However, little is known about the precise mechanism of negative regulation of Slt2.

Since the completion of the genome sequence of *S. cerevisiae*, genomic scale analyses have provided a wealth of information to experimentally examine the potential function of newly identified proteins and their interactions. One of the reports analyzing global protein-protein interactions using two-hybrid screens suggested an interaction between the Slt2 kinase and Yil113w, a potential dual specificity phosphatase (30). In addition, the genome-wide analysis of genomic expression patterns in response to environmental stresses showed induction of *YIL113W* mRNA expression under stress conditions (31). In this study, we demonstrated that Yil113w (renamed as Sdp1 for stress-inducible dual specificity phosphatase) dephosphorylates Slt2 *in vivo* and *in vitro*. The function and Msn2/4-dependent stress-induced expression of *SDP1* demonstrate a role for Sdp1 in negative regulation of Slt2 following stress activation.

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions—All the kinase (*slt2*Δ and *hog1*Δ) and phosphatase (*ptp2*Δ, *ptp3*Δ, *msg5*Δ, and *sdp1*Δ) deletion strains used in this study are strains derivative of BY4741 (MATa *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) or homozygous diploids in the BY4743 background (MATa/α *his3Δ1 leu2Δ0 ura3Δ0*) (Research Genetics). The JH20 (*ptp2*Δ::kanMX4 *sdp1*Δ::His3MX6) and JH21 (*SDP1*-13Myc:His3MX6) strains were generated from BY4741 *ptp2*Δ and BY4741, respectively, by a PCR-based integration procedure (32). The *msn2msn4* and *rlm1*Δ strains are derived from W303–1A (MATa *ade2–1 can1–100 his3–11, 15 leu2–3,-112 trp1–1 ura3–1*). The *msn2msn4* mutant (33) was kindly provided by F. Estruch, and the *rlm1*Δ strain (*rlm1*Δ::kanMX6) was generated by PCR-mediated gene deletion method (32). Yeast cultures were grown in YPD medium (1% yeast extract, 2% bactopeptone, 2% dextrose) or synthetic complete (SC) medium lacking proper amino acids to maintain plasmids.

Plasmids—Plasmid pNV7-*MKK1*^{p386} (P_{GAL1}*MKK1*^{p386}) (24) was kindly provided by K. Irie and K. Matsumoto, and plasmid YEp352-*SLT2-3HA* (25) was a kind gift from M. Snyder. Plasmid pRS415-*SDP1* was generated by cloning a 1.6-kb PCR product, which contains *SDP1* promoter region up to −1000 and *SDP1* open reading frame with a *Not*I restriction site before the stop codon, into pRS415 lacking the *Not*I site. To generate plasmid pRS415-*SDP1-GFP*, a 750-bp *Not*I fragment of GFP derived from pSF1-GFP1 was inserted into the *Not*I site of pRS415-*SDP1*. A *Bam*HI/*Sal*I fragment from pRS415-*SDP1* and a *Bam*HI fragment from pRS415-*SDP1-GFP* were cloned into pRS425 to generate pRS425-*SDP1* and pRS425-*SDP1-GFP*, respectively. Plasmid pRS425-*SLT2-GFP* containing *SLT2* open reading frame and promoter region up to −1000 was constructed with the same strategy for generation of pRS425-*SDP1-GFP*.

Immunoblot Analysis—Yeast extracts for immunoblot analysis of Slt2 were prepared as follows. Yeast cells were grown to A_{600} of 0.7 in YPD medium at 25 °C and then shifted to 39 °C for heat shock. Cells were harvested by adding an equal volume of ice, washed with ice-cold water, and then frozen in dry ice. Cell pellet from 30 ml of culture was broken by vortexing with glass beads in 300 μl of lysis buffer (50 mM Tris-HCl (pH 7.5), 10% glycerol, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 15 mM $Na_2H_2P_2O_7$, 15 mM *p*-nitrophenyl phosphate, 0.2 mM sodium orthovanadate) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml pepstatin, 10 μg/ml aprotinin). 100–150 μg of proteins were subjected to 10% SDS-PAGE followed by electrotransfer to nylon membrane. Phosphorylated Slt2 was detected with anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) antibody (New England Biolabs), and Slt2 was detected with anti-Slt2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunoblots were developed with horseradish peroxidase-conjugated secondary antibody and Super Signal chemiluminescent substrate kit from Pierce.

In Vitro Dephosphorylation of Slt2—The *SDP1* open reading frame was cloned in pGEX-3X GST gene fusion vector (Amersham Biosciences), and GST-Sdp1 was purified from *Escherichia coli* BL21(DE3) by a standard protocol. GST-Sdp1 fusion protein was cleaved with factor Xa protease, and the GST protein was removed by incubation with glutathione-agarose (Sigma). The BY4741 *slt2*Δ cells containing YEp352-*SLT2-3HA* were grown in SC-Ura medium at 25 °C to A_{600} of 0.7 and then heat-shocked at 39 °C for 1 h to activate Slt2. 1 mg of cell extracts was incubated with 2 μg of anti-HA antibody (3F10; Roche Molecular Biochemicals) for 2 h at 4 °C, followed by further incubation with 40 μl of 50% slurry of protein A-Sepharose (Sigma) for 2 h. The precipitates were washed four times with 1 ml of lysis buffer and three times with phosphatase buffer (50 mM Tris (pH 7.4) and 10 mM dithiothreitol) and then resuspended in 100 μl of phosphatase buffer. 20 μl of precipitated material was incubated with 1 μg of Sdp1 protein at 30 °C for 30 min in the presence or absence of 2 mM sodium orthovanadate. The reaction was terminated by the addition of SDS sample buffer and subjected to anti-phospho-p42/44 immunoblotting to detect phosphorylated Slt2–3HA. The same membrane was stripped and reprobed with anti-HA antibody to determine the levels of Slt2–3HA.

Co-immunoprecipitation—Co-immunoprecipitation of Sdp1–13Myc and Slt2–3HA was carried out as follows. JH21 strain, in which the *SDP1* gene was replaced by *SDP1*-13Myc, was transformed with YEp352-*SLT2–3HA* or YEp352 plasmid. Cells were grown in SC-Ura medium up to A_{600} of 0.7 at 25 °C and then shifted to 39 °C for 1 h. Both heat-treated and untreated cells were broken in immunoprecipitation buffer (50 mM HEPES (pH 7.6), 100 mM NaCl, 0.5% Nonidet P-40) with protease inhibitors. 1 mg of cell extracts was immunoprecipitated with anti-HA antibody and protein A-Sepharose. The precipitates were washed five times with 1 ml of immunoprecipitation buffer, resuspended in 50 μl of 2× SDS sample buffer, and boiled for 5 min. The eluted materials were subjected to immunoblot analysis with anti-HA antibody or anti-Myc (9E10; Roche Molecular Biochemicals) antibody.

Northern Blot Analysis—Yeast cells were grown in YPD medium to A_{600} of 0.7 at 30 °C and shifted to 39 °C for heat shock. For oxidative stress or osmotic stress, a final concentration of 0.3 mM H_2O_2 or an equal volume of YPD containing 2 M sorbitol was added to the culture and incubated further at 30 °C. PCR-amplified open reading frames of *SLT2*, *MSG5*, *SDP1*, and *ACT1* were used as probes for Northern blotting. For the detection of *PTP2* and *PTP3* mRNA, 0.7-kb *Spe*I/*Nco*I and 0.9-kb *Xba*I/*Hin*dIII internal fragments were used, respectively.

Fluorescence Microscopy—To localize Sdp1 and Slt2, BY4743 diploid *sdp1*Δ cells containing pRS425-*SDP1-GFP* and *slt2*Δ cells containing pRS425-*SLT2-GFP* were grown in SC-Leu medium. 10 μg/ml 4′,6-diamidino-2-phenylindole was added for nuclear staining, and the fluorescence images were detected using a Nikon Eclipse E800 fluorescence microscope equipped with a Hamamatsu ORCA-2 cooled CCD camera.

RESULTS

Sdp1 Phosphatase Regulates the Slt2 Cell Integrity Pathway—To test the possible role of the Sdp1 (Yil113w) dual specificity phosphatase in regulation of Slt2 kinase, we examined whether deletion or overexpression of *SDP1* could affect growth defects caused by hyperactivation of the Slt2 pathway. Overexpression of the Mkk1^{p386} allele, which was originally identified as a suppressor of the cell lysis phenotype of a *bck1*Δ strain, inhibits growth by constitutive activation of the downstream MAPK Slt2 (24, 34). *S. cerevisiae* strains were transformed with plasmid pNV7-*MKK1*^{p386} (P_{GAL1}*MKK1*^{p386}) (24), in which the *MKK1*^{p386} allele is under the control of the *GAL1* promoter, and tested for growth on medium containing glucose (repressing) or galactose (inducing) (Fig. 1*A*). A growth defect observed on galactose medium by *MKK1*^{p386} overexpression was exacerbated in *ptp2*Δ and *msg5*Δ deletion strains compared with the wild type as previously reported (8, 24). Although a previous report indicated no difference in growth between wild type and *ptp3*Δ strains upon *MKK1*^{p386} overexpression (8), we could observe a modest growth defect in the *ptp3*Δ strain under our experimental conditions, perhaps reflecting a minor contribution of Ptp3 in Slt2 dephosphorylation compared with Ptp2. The effect of *SDP1* deletion on the *MKK1*^{p386} phenotype was weaker than that of the *PTP3* deletion, but the *sdp1*Δ strain also showed poorer growth than the wild type on galactose medium with virtually indistinguishable growth on glucose medium.

As a corollary experiment, we tested whether overexpression of Sdp1 could suppress the slow growth phenotype of yeast cells expressing Mkk1^{p386}. Expression of *SDP1* both on a low copy number plasmid (pRS415) and a high copy number plasmid

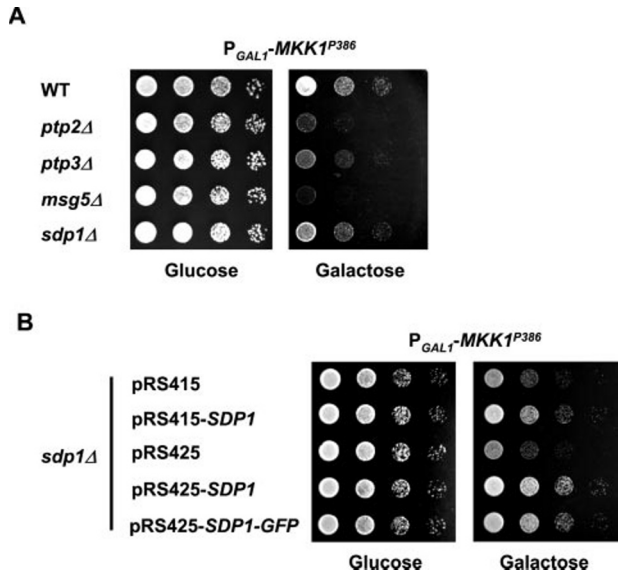

FIG. 1. **Effects of the Sdp1 expression levels on growth defects caused by overexpression of constitutively active Mkk1.** *A*, deletion of *PTP2*, *PTP3*, *MSG5*, and *SDP1* intensifies the growth defects caused by overexpression of constitutively active Mkk1. Wild type (*WT*) (BY4741), *ptp2*Δ, *ptp3*Δ, *msg5*Δ, and *sdp1*Δ strains carrying pNV7-*MKK1*^{p386} (P_{GAL1}-*MKK1*^{p386}) were grown in selective medium containing 2% raffinose. 10-Fold serial dilutions of A_{600} of 1.0 cells were spotted on selective medium containing glucose or galactose. *B*, overexpression of Sdp1 suppresses the growth defects caused by overexpression of Mkk1^{p386}. The *sdp1*Δ strains carrying pNV7-*MKK1*^{p386} and the indicated plasmids were grown in selective medium containing raffinose, and growth on selective medium containing glucose or galactose was examined as described for *A*.

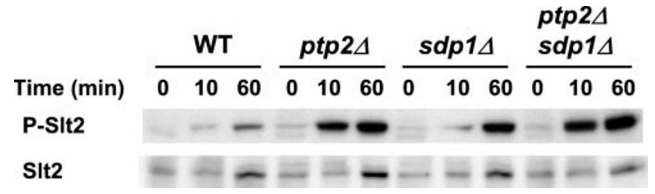

FIG. 2. **The Sdp1 regulates the level of Slt2 phosphorylation in response to heat shock.** Wild type (*WT*) (BY4741) and *sdp1*Δ, *ptp2*Δ, and *sdp1*Δ*ptp2*Δ strains were grown in YPD medium to midlog phase at 25 °C and then heat-shocked at 39 °C for the indicated times. The levels of phospho-Slt2 and total Slt2 were detected by immunoblot analysis with anti-phospho-p42/44 MAPK antibody and anti-Slt2 antibody, respectively.

FIG. 3. **Sdp1 dephosphorylates Slt2 *in vitro*.** The *slt2*Δ cells containing YEp352-*SLT2–3HA* were grown at 25 °C and shifted to 39 °C for 1 h. Slt2–3HA was immunoprecipitated with anti-HA antibody and then incubated with 1 μg of Sdp1 purified from *E. coli* at 30 °C for 30 min in the presence or absence of 2 mM sodium orthovanadate. The levels of phospho-Slt2–3HA and Slt2–3HA were detected by immunoblotting with anti-phospho-p42/44 antibody and anti-HA antibody, respectively.

(pRS425) suppressed Mkk1^{p386}-mediated growth defects in *sdp1*Δ strains (Fig. 1*B*). Expression of an Sdp1-GFP fusion protein (pRS425-*SDP1-GFP*) in the *sdp1*Δ strain restored growth on galactose medium to the same extent of pRS425-*SDP1* expression.

Sdp1 Dephosphorylates Slt2 in Vivo and in Vitro—Since expression of Sdp1 can reverse growth defects associated with hyperactivation of the Slt2 pathway and Slt2 is activated by phosphorylation, we investigated the possibility that phosphorylated Slt2 might be a direct target for the action of the Sdp1 putative phosphatase. To this end, we compared the heat-induced phosphorylation state of Slt2 in wild type and *sdp1*Δ strains using anti-phospho-p44/42 MAPK antibody, which can recognize phosphorylated Thr^{190} and Tyr^{192} in the activation loop of Slt2 (10) (Fig. 2). When wild type cells were shifted from 25 to 39 °C, phosphorylation of Slt2 was weakly detected after 10 min and reached higher levels after 60 min. In comparison, the *ptp2*Δ, *sdp1*Δ, and *ptp2*Δ*sdp1*Δ mutants showed slightly higher basal levels and significantly higher heat-induced levels of phosphorylation of Slt2. Phosphorylation of Slt2 was highly induced 10 min after heat shock in *ptp2*Δ and *ptp2*Δ*sdp1*Δ compared with the wild type or *sdp1*Δ strain. The *ptp2*Δ*sdp1*Δ strain showed slightly higher levels of Slt2 phosphorylation after heat shock than *ptp2*Δ. Although previous reports using an Slt2-HA overexpression vector showed no induction of Slt2 protein levels in response to heat shock (8, 35), we reproducibly detected significant elevation of endogenous Slt2 steady state protein levels 60 min after heat shock. This induction of Slt2 protein levels was delayed as compared with the induction of *SLT2* mRNA following heat shock (Fig. 6*A*), which was detectable within 10 min.

Because *SDP1* status affected the steady state phosphorylation of Slt2 kinase, we tested whether recombinant Sdp1 could dephosphorylate Slt2 *in vitro*. Sdp1 purified from *E. coli* showed phosphatase activity toward the small-molecule phosphatase substrate *p*-nitrophenyl phosphate (data not shown). Furthermore, Sdp1 dephosphorylated Slt2–3HA that had been immunoprecipitated from heat-shocked yeast cells (Fig. 3). Incubation with sodium orthovanadate, an inhibitor of protein-tyrosine phosphatases, inhibited dephosphorylation of Slt2–3HA by Sdp1 *in vitro*. Taken together, these results suggest that Sdp1 functions in the Slt2 pathway by direct dephosphorylation of Slt2 kinase.

Sdp1 Interacts with Slt2 in Vivo—Data described above suggest a functional interaction between the Sdp1 phosphatase and the Slt2 kinase. Although genome-wide two-hybrid analysis suggested an interaction between a Gal4 activation domain (GAD) fusion of Slt2 and Gal4 DNA binding domain (GBD) fusion of Sdp1 (30), it was necessary to confirm their interaction to rule out the possibility of a false positive interaction. *S. cerevisiae* SFY526 strain containing an integrated *GAL1-lacZ* reporter was transformed with plasmids expressing GBD-Sdp1 and GAD-Slt2 or VP16-Slt2 fusion proteins, and the interactions were detected by β-galactosidase assays (Fig. 4*A*). Although the overall activity was low, transformants expressing GBD-Sdp1 and GAD-Slt2 (1.3 Miller units) or GBD-Sdp1 and VP16-Slt2 (2.3 Miller units) showed higher activity than the cells containing GBD-Sdp1 alone (0.1 Miller units) or GAD-Slt2 alone (0.2 Miller units), suggesting an interaction between Slt2 and Sdp1.

We also tested the interaction between Sdp1 and Slt2 by co-immunoprecipitation experiments from yeast cell extracts. For this purpose, we constructed a genomic tagged version of Sdp1 encoding 13Myc epitopes at the carboxyl terminus. The 13Myc-tagged Sdp1 was fully functional as ascertained by the resistance of cells expressing Sdp1–13Myc to toxicity due to Mkk1^{p386} overexpression (data not shown). Cells expressing Sdp1–13Myc were transformed with YEp352-*SLT2–3HA* or vector control, and the transformants were grown at 25 °C and heat-shocked at 39 °C for 1 h before harvest and protein extraction under native conditions. Slt2–3HA was immunoprecipitated with anti-HA antibody and immunoblotted with anti-Myc antibody. This experiment revealed co-immunoprecipitation of Sdp1–13Myc with Slt2–3HA (Fig. 4*B*). The

strength of the interaction, at least by this analysis, was not responsive to heat shock.

The Localization of Sdp1 Changes upon Heat Shock—To localize Sdp1 within yeast cells, we expressed a functional Sdp1-GFP fusion protein in a diploid *sdp1*Δ strain. At 25 °C, Sdp1-GFP was localized in both the nucleus and cytoplasm with slight accumulation in the nucleus (Fig. 5*A*). Upon heat shock, the Sdp1-GFP fusion was observed in punctate spots throughout the cells. This relocalization of Sdp1-GFP was observed within 5 min after heat shock, and after 30 min of heat shock, the localization pattern was more intense and punctate. The punctate Sdp1-GFP spots were redistributed evenly throughout the cell when the heat-shocked cells were shifted back to 25 °C for 20 min. This translocation of Sdp1 was quite specific to heat shock and Sdp1-GFP, since we could not detect changes in Sdp1-GFP localization after treatment with 0.3 mM H_2O_2 or 1 M sorbitol (data not shown). We also localized Slt2 using a functional Slt2-GFP fusion, which can complement the temperature-sensitive phenotype of an *slt2*Δ mutant. In accordance with the previous report localizing Slt2-HA (35), Slt2-GFP was concentrated in the nucleus and also located in the cytoplasm at 25 °C (Fig. 5*B*). Although a previous report showed more uniform distribution of Slt2-HA between the cytoplasm and the nucleus after heat shock (35), the Slt2-GFP fusion showed little change in localization 30 min after heat shock.

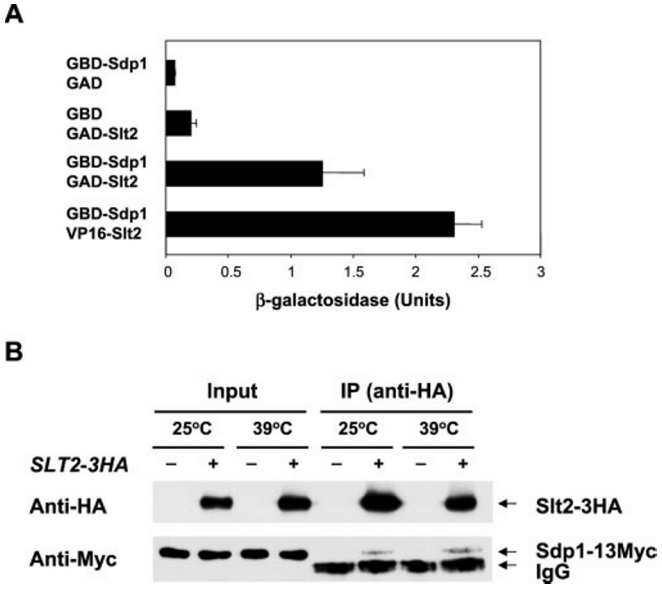

FIG. 4. **Interaction of Sdp1 and Slt2 *in vivo*.** *A*, two-hybrid interaction of Sdp1 and Slt2. SFY526 transformants expressing the indicated proteins were grown to midlog phase in selective medium, and β-galactosidase activity was measured in permeabilized cells. *B*, co-immunoprecipitation of Slt2 and Sdp1 *in vivo*. JH21 strain expressing Sdp1–13Myc was transformed with YEp352 or YEp352-*SLT2–3HA*. The transformants were grown in selective medium at 25 °C and heat-shocked at 39 °C for 1 h. Cell extracts from the control and heat-shocked cells were immunoprecipitated with anti-HA antibody, and samples were analyzed by immunoblotting with anti-HA and anti-Myc antibodies.

Differential Expression of SLT2 and Phosphatase Genes in Response to Stress Conditions—Slt2 is regulated by at least four protein phosphatases, Ptp2, Ptp3, Msg5, and Sdp1. These phosphatases show diverse specificity toward other MAPKs. Ptp2 and Ptp3 are also involved in dephosphorylation of Fus3 and Hog1, and Msg5 also dephosphorylates Fus3 and Kss1 (6, 7, 30). In addition to specific interactions between the phosphatases and kinases, the abundance of individual phosphatases is likely to be an important factor determining the specificity of the phosphatases under distinct stress conditions that activate specific MAPKs.

Since the *SDP1* and *PTP2* promoters contain stress response elements, which are the binding sites for the general stress transcription factors Msn2/4 (36), we investigated whether Msn2/4 are involved in the expression of *SLT2* and phosphatase genes under a number of stress conditions. We compared stress-inducible gene expression patterns between the W303–1A wild type strain and an isogenic *msn2msn4* mutant (Fig. 6*A*). As previously established, *SLT2* and *PTP2* steady state mRNA levels were induced by heat shock (8, 27). These two genes showed slow induction kinetics compared with *SDP1* induction, which peaked at 10 min after heat shock. The *SLT2*, *PTP2*, and *SDP1* genes were also induced by H_2O_2 treatment. In contrast, expression of *PTP3* and *MSG5* was transiently decreased upon heat shock, and there was little change in expression by H_2O_2 treatment. The *SLT2* and all all the phos-

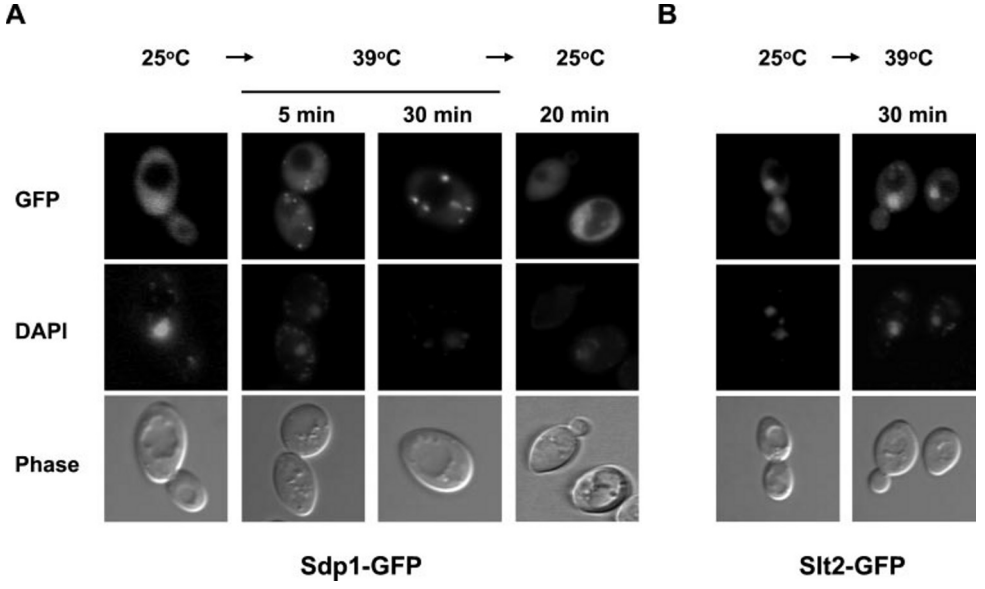

FIG. 5. **Subcellular localization of Sdp1 and Slt2 in response to heat shock.** *A*, localization of Sdp1-GFP. The homozygote diploid (BY4743) *sdp1*Δ strain carrying pRS425-*SDP1-GFP* was grown in selective medium at 25 °C, heat-shocked at 39 °C for 30 min, and then shifted back to 25 °C for 20 min. GFP and 4′,6-diamidino-2-phenylindole (*DAPI*) signals were detected with fluorescence microscopy. *B*, localization of Slt2-GFP. The homozygote diploid *slt2*Δ strain carrying pRS425-*SLT2-GFP* was grown in selective medium at 25 °C and heat-shocked at 39 °C for 30 min.

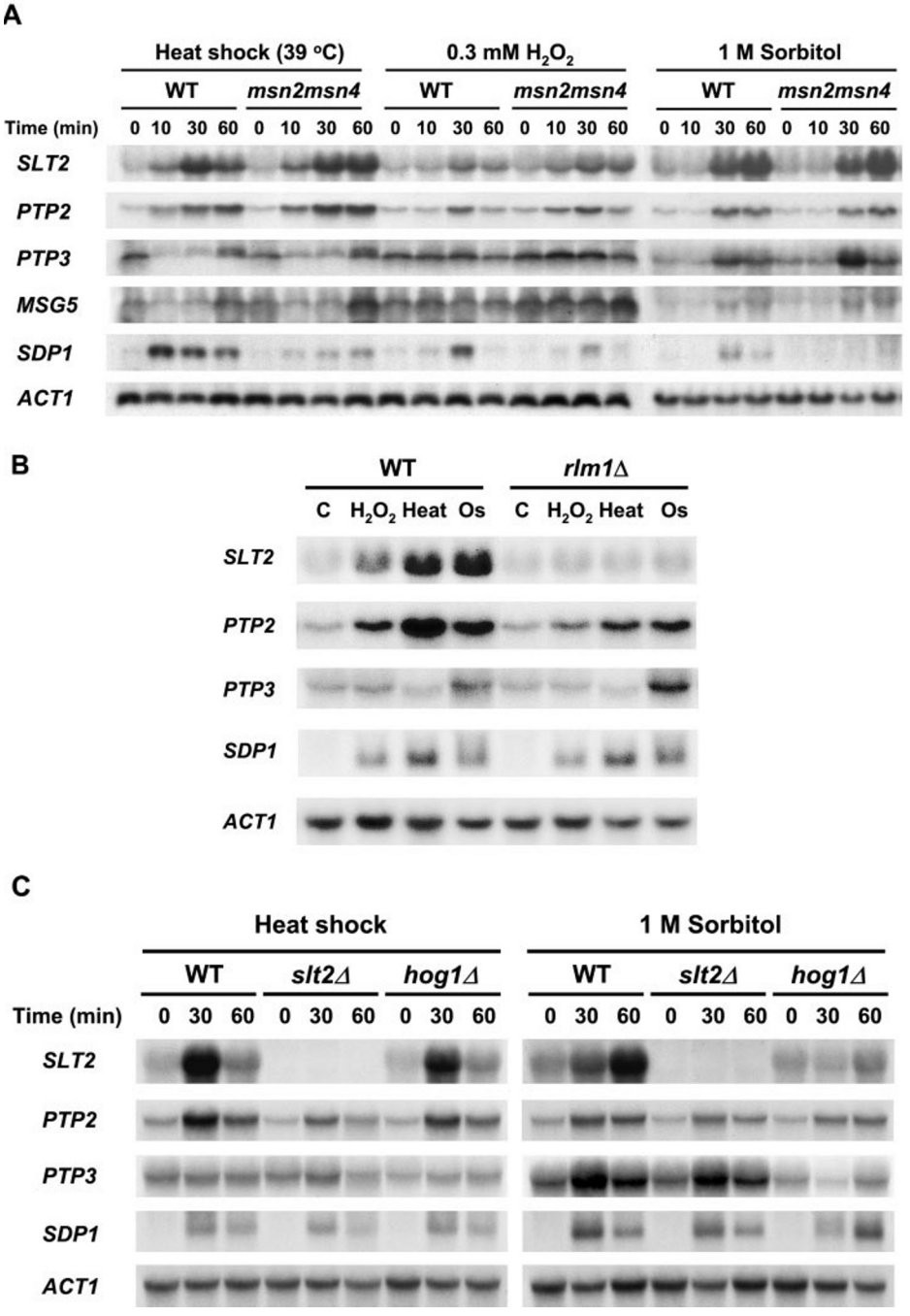

FIG. 6. **Transcriptional regulation of the *SLT2* and phosphatase genes in response to various stresses.** *A*, Msn2/4-dependent induction of *SDP1* mRNA under stress conditions. Wild type (*WT*) (W303–1A) and *msn2msn4* strains, grown in YPD medium to A_{600} of 0.7 at 30 °C, were shifted to 39 °C or treated with 0.3 mM H_2O_2 or 1 M sorbitol. Samples were taken at the indicated times, and the levels of *SLT2*, *PTP2*, *PTP3*, *MSG5*, *SDP1*, and *ACT1* transcripts were detected by Northern blot analysis. *B*, Rlm1-dependent induction of *SLT2* and *PTP2* upon stresses. Northern blot analysis was done in wild type (W303–1A) and *rlm1*Δ strains treated with H_2O_2, heat shock, or 1 M sorbitol (*Os*) for 30 min. *C*, Hog1-dependent induction of *SLT2* by osmotic stress. Northern blot analysis was done in stress-treated wild type (BY4741), *slt2*Δ, and *hog1*Δ strains.

phatase genes tested (PTP2, PTP3, MSG5, and SPP1) were induced by osmotic stress 30 min after treatment. Only *SDP1* was dependent on Msn2/4 for induction by heat, H_2O_2, and osmotic stress via the administration of 1 M sorbitol. These data suggest differential regulation of *SLT2* and phosphatase genes under different stress conditions.

Previous studies have shown that the Rlm1 transcription factor, which is activated by Slt2-dependent phosphorylation, is responsible for *SLT2* mRNA induction in response to heat shock (24, 27, 37). In addition, it has been shown that induction of *PTP2* by heat shock is dependent on Slt2 (8). To examine the contribution of Rlm1 in the activation of *SLT2* and phosphatase genes in response to oxidative stress, osmotic stress, and heat shock, we compared stress-inducible expression of these genes in isogenic wild type and *rlm1*Δ strains (Fig. 6*B*). *SLT2* induction by all of the stresses tested, H_2O_2, heat shock, and osmotic stress, was not observed in the *rlm1*Δ strain, whereas *PTP2* induction was largely, although not completely, dependent on Rlm1. Expression of *PTP3* and *SDP1* in response to stresses was not significantly changed in the *rlm1*Δ strain. The residual heat shock induction of *SDP1* observed in *msn2msn4* (Fig. 6*A*) was still detected in an *msn2 msn4 rlm1*Δ mutant strain (data not shown). Therefore, the stress-inducible expression of *SDP1* is independent of the Rlm1 transcription factor but partially dependent on Msn2/4-mediated activation in response to heat shock, H_2O_2, and osmotic stress.

It has been previously demonstrated that Slt2 and Hog1 respond in opposite ways to osmotic changes. Slt2 is transiently activated in response to hypoosmolarity, whereas Hog1 is activated by hyperosmolarity (16). Since *SLT2* mRNA was induced by 1 M sorbitol in an Rlm1-dependent manner, we hypothesized that there might be an upstream regulator other than Slt2 to activate Rlm1 in response to hyperosmotic stress. Mlp1 was identified as an Rlm1-interacting protein that has

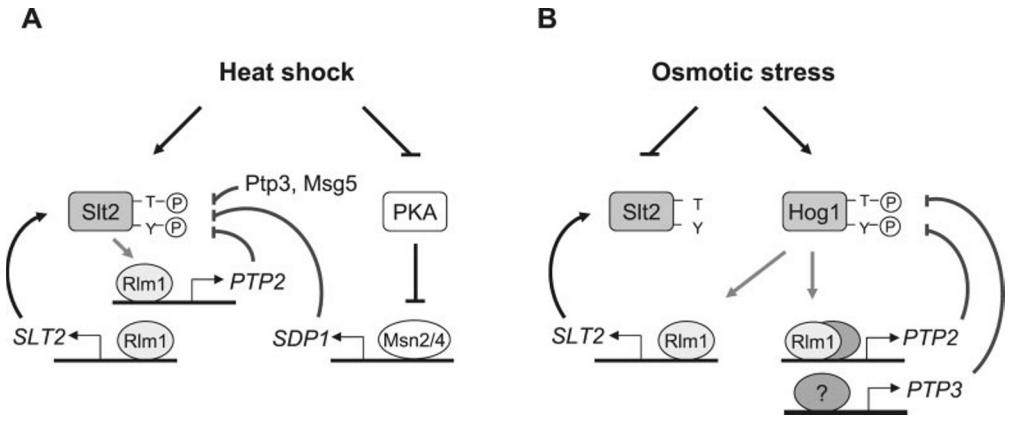

FIG. 7. **Models for regulation of Slt2 activity and gene expression.** *A*, regulation of Slt2 under heat shock conditions. Slt2 is activated by heat shock and activates Rlm1 to induced expression of *PTP2* and *SLT2*. Expression of *SDP1* is induced by Msn2/4, which are under the control of the cAMP-dependent kinase PKA. The Ptp2 and Sdp1 down-regulate Slt2 by dephosphorylation. Although expression of Ptp3 and Msg5 is not induced by heat shock, they are also involved in inactivation of Slt2. *B*, regulation of Slt2 and Hog1 under hyperosmotic conditions. Hyperosmotic stress activates Hog1, while it inactivates Slt2. Activated Hog1 regulates Rlm1 by a yet unknown mechanism to activate expression of *SLT2* and *PTP2*. *PTP2* expression may be also regulated by other unknown transcription factors. Expression of *PTP3* is induced by a Hog1-dependent transcription factor yet unidentified. Ptp2 and Ptp3 inactivate Hog1 by dephosphorylating a phosphotyrosine residue, forming feedback regulation.

high homology with Slt2. However, induction of *SLT2* mRNA by any of the stress conditions was not changed in an *mlp1*Δ mutant strain (data not shown). In a *hog1*Δ strain, *SLT2* mRNA induction by 1 M sorbitol was significantly reduced without any effect on induction by heat shock (Fig. 6*C*). This result suggests that Hog1 might be involved in Rlm1 activation in response to hyperosmotic stress. The induction of *PTP2* by heat shock was reduced in *slt2*Δ, reflecting a contribution of Rlm1 on heat shock-induction of *PTP2* (Fig. 6*B*). Previously, it has been shown that induction of *PTP2* and *PTP3* mRNA by 0.4 M NaCl is dependent on Hog1 (6). In accordance with the previous report, *PTP3* induction by 1 M sorbitol was dependent on Hog1. However, about 2-fold induction of *PTP2* by 1 M sorbitol was still observed in *slt2*Δ or *hog1*Δ strains with a slight reduction in the expression levels. Although genome-wide analysis showed that NaCl and sorbitol gave the similar profiles of gene induction (38), using different inducers and a time course evaluation might give distinct results for Hog1-dependent induction of *PTP2*. The upstream regulators responsible for *SLT2* and *PTP2* induction by oxidative stress are not clear yet. We could not detect significant changes in H_2O_2 induction of *SLT2* in *hog1*Δ and *PTP2* induction in *slt2*Δ or *hog1*Δ strains (data not shown). It has been shown that Mkp1, an Slt2 homologue of *Pneumocystis carinii*, can be activated by H_2O_2 *in vitro* (39). However, it remains to be ascertained whether Slt2 can be activated by H_2O_2 and whether this activation is responsible for H_2O_2 induction of *PTP2* and *SLT2*. Therefore, differential expression of phosphatase mRNAs under stress conditions would provide feedback regulation of *SLT2* under specific stress conditions activating Slt2. In addition, Msn2/4-dependent expression of *SDP1* suggests cross-talk between the Slt2 and cAMP-PKA pathways, whereas Hog1-dependent induction of *SLT2* under hyperosmotic conditions might establish linkage between the Slt2 and Hog1 MAPK pathways in transcription.

DISCUSSION

The Sdp1 Dual Specificity Phosphatase Inactivates Slt2 by Direct Dephosphorylation—The Slt2 MAPK pathway is essential to maintain cell wall structure during vegetative growth and mating and in response to environmental stresses that perturb cell wall integrity. Therefore, Slt2 activity must be orchestrated with cellular processes such as the cell cycle, pheromone responses, and stress responses.

Including the Sdp1 dual specificity phosphatase, which was demonstrated to negatively regulate Slt2 in the present study, four phosphatases have been identified as regulators of Slt2. Two protein-tyrosine phosphatases, Ptp2 and Ptp3, and two dual specificity phosphatases, Msg5 and Sdp1, appear to have redundant roles as well as specific roles in the regulation of Slt2. All of the phosphatase deletion strains tested, especially *ptp2*Δ*ptp3*Δ (8) and *msg5*Δ (10), showed higher basal phosphorylation levels of Slt2 than the wild type, suggesting a role for these phosphatases in regulating basal activity of Slt2. In addition, heat-induced Slt2 phosphorylation was also increased in *ptp2*Δ, *sdp1*Δ (Fig. 2), *ptp2*Δ*ptp3*Δ (8), and *msg5*Δ (10) strains, implying the potential involvement of these phosphatases in the down-regulation of Slt2 activity for adaptation after stress. Since Slt2 can be activated by various stress stimuli other than heat shock, the role of the four phosphatases might be differentiated under specific stress conditions. It remains to dissect out specific roles of these phosphatases in regulation of Slt2 and other target kinases.

It has been shown that protein-protein interaction through amino-terminal noncatalytic domains of Ptp2 and Ptp3 determines their substrate specificity toward Hog1 and Fus3 (40). In addition, the localization of phosphatases could affect their specificity toward various MAPKs. Ptp2 is nuclear, whereas Ptp3 is cytoplasmic and excluded from the nucleus (8). Moreover, the Ptp2 and Ptp3 can regulate Hog1 localization by tethering Hog1 in the nucleus and cytoplasm, respectively (9). At 25 °C, Sdp1-GFP was observed throughout the cell with slightly enhanced nuclear localization. Interestingly, Sdp1-GFP showed rapid translocation to punctate spots after heat shock, implying association of Sdp1 with subcellular organelles or with other proteins. Identification of Sdp1 substrates other than Slt2 will help to identify the location of Sdp1 after heat shock, and the significance of the translocation event.

Expression of PTP2 and SDP1 mRNA upon Heat Shock Forms Feedback Regulation of the Slt2 Pathway and Cross-talk between Slt2 and cAMP-PKA Pathways—The expression of many phosphatases that act upon MAPKs is under the control of their target MAPKs or upstream signals activating MAPKs, forming feedback regulation loops (3). In accordance with the previous report showing Slt2-dependent induction of *PTP2* by heat shock (8), we showed that Slt2 and its downstream transcription factor Rlm1 is largely involved in heat shock induction of *PTP2*. *SDP1* was induced by various stress conditions such as heat shock, oxidative stress, and osmotic stress in an

Msn2/4-dependent manner but independent of the Slt2 pathway. Msn2/4 transcription factors regulate genes containing stress response elements, and their activity is negatively regulated by the cAMP-PKA pathway, which is involved in nutrient signaling (41). The putative sensors of the Slt2 pathway, Wsc1 and Wsc2, were isolated as multicopy suppressors of heat shock sensitivity of *ira1*Δ strain in which hyperactivation of Ras causes an increase in cAMP production, suggesting possible cross-talk between the Slt2 and the cAMP-PKA pathways (20). Taken together, under heat shock conditions, Rlm1-dependent induction of *PTP2* forms feedback regulation of Slt2 activity, whereas regulation of Slt2 activity by Sdp1 whose expression is dependent on Msn2/4 would provide linkage between the Slt2 and cAMP-PKA pathways (Fig. 7*A*).

Hog1 Regulates Rlm1-dependent SLT2 Expression upon Osmotic Stress—It has been known that Rlm1, whose activity is regulated by Slt2, mediates heat shock induction of Slt2-regulated genes including *SLT2* itself (27). Most of the Rlm1-regulated genes identified by genome-wide analysis encode cell wall proteins or enzymes involved in cell wall biosynthesis (27). Furthermore, although it remains to be confirmed, putative Rlm1-binding consensus elements are identified on the promoters of genes involved in Slt2 pathway such as *MID2*, *SAC7*, *BCK1*, *MKK1*, and *RLM1* itself. We demonstrated here that Rlm1 is responsible for not only heat shock induction but also H_2O_2 and osmotic stress induction of *SLT2*. We also showed that Hog1 kinase is involved in induction of *SLT2* by hyperosmotic stress, suggesting cross-talk between Hog1 and Slt2 kinase pathways. Hog1 and Slt2 kinases are activated by hyperosmolarity and hypoosmolarity, respectively. Although Hog1 and Slt2 kinases are regulated in opposite directions by changes in external osmolarity, it is likely that they are coordinately regulated. Dephosphorylation of Hog1 by hypoosmotic stress has been shown to be dependent on Slt2 pathway (16). The biological significance of Hog1-dependent induction of *SLT2* is not yet clear; however, it might reflect requirements for cell wall changes after adaptation to hyperosmotic stress. Induction of some cell surface proteins or cell wall biosynthetic enzymes by hyperosmotic stress supports this idea (38).

Hyperosmotic stress induction of *SLT2* is dependent on Hog1 kinase and the Rlm1 transcription factor. These data suggest that the Hog1 kinase pathway may regulate Rlm1 activity under high osmolarity conditions (Fig. 7*B*). Rlm1 is likely to be partially involved in osmotic induction of *PTP2* to form feedback regulation of Hog1. Osmotic stress induction of *PTP3* might be mediated by yet unidentified transcription factors that are regulated by Hog1. Rlm1 is a member of the MADS (Mcm1, Agamous, Deficiens, SRF) box family of transcription factors, which have a conserved amino-terminal MADS box DNA binding domain (24). One of the characteristics of MADS box proteins is their interaction with co-regulators to regulate gene expression. Two-hybrid screening identified Mlp1, a homologous protein to Slt2, as an Rlm1-interacting protein (24). Although there is no evidence that Rlm1 interacts with other proteins than Slt2 and Mlp1, it is still possible that additional accessory proteins are involved in regulation of Rlm1 activity under different conditions. Further studies are necessary to determine whether Hog1 could regulate Rlm1 directly or indirectly by regulating interacting partners and how Rlm1 is differentially regulated under heat shock and osmotic stress conditions.

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