

Targeting the MEF2-Like Transcription Factor Smp1 by the Stress-Activated Hog1 Mitogen-Activated Protein Kinase

Eulàlia de Nadal, Laura Casadomé, and Francesc Posas*

Cell Signaling Unit, Departament de Ciències Experimentals i de la Salut, Universitat Pompeu Fabra, Barcelona E-08003, Spain

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Exposure of *Saccharomyces cerevisiae* to increases in extracellular osmolarity activates the stress-activated Hog1 mitogen-activated protein kinase (MAPK), which is essential for cell survival upon osmotic stress. Yeast cells respond to osmotic stress by inducing the expression of a very large number of genes, and the Hog1 MAPK plays a critical role in gene transcription upon stress. To understand how Hog1 controls gene expression, we designed a genetic screen to isolate new transcription factors under the control of the MAPK and identified the MEF2-like transcription factor, Smp1, as a target for Hog1. Overexpression of *SMP1* induced Hog1-dependent expression of osmoreponsive genes such as *STL1*, whereas *smp1* Δ cells were defective in their expression. Consistently, *smp1* Δ cells displayed reduced viability upon osmotic shock. In vivo coprecipitation and phosphorylation studies showed that Smp1 and Hog1 interact and that Smp1 is phosphorylated upon osmotic stress in a Hog1-dependent manner. Hog1 phosphorylated Smp1 in vitro at the C-terminal region. Phosphorylation of Smp1 by the MAPK is essential for its function, since a mutant allele unable to be phosphorylated by the MAPK displays impaired stress responses. Thus, our data indicate that Smp1 acts downstream of Hog1, controlling a subset of the responses induced by the MAPK. Moreover, Smp1 concentrates in the nucleus during the stationary phase, and the lack of *SMP1* results in cells that lose viability in the stationary phase. Localization of Smp1 depends on *HOG1*, and consistently, *hog1* Δ cells also lose viability during this growth phase. These data suggest that Smp1 could be mediating a role for the Hog1 MAPK during the stationary phase.

Mitogen-activated protein kinase (MAPK) cascades are common signaling modules found in both higher and lower eukaryotic cells. Activation of a MAPK results in modification of a set of target proteins, often transcription factors, that allow the generation of appropriate cellular responses to an external stimulus. Stress-activated protein kinases (SAPKs) are a subset of MAPKs activated by environmental and genotoxic stresses (reviewed in references 9 and 20). A prototype of the SAPK family is the yeast p38-related MAPK, Hog1, which specifically responds to increased extracellular osmolarity and is required for cell survival under these conditions.

Activation of the yeast Hog1 MAPK induces diverse osmoadaptive responses, such as regulation of gene expression. Genome-wide transcriptional analyses showed that a great number of genes are regulated by osmotic stress in a *HOG1*-dependent manner. Among the genes under the control of Hog1 are genes that encode proteins implicated in carbohydrate metabolism, general stress protection, protein production, and signal transduction (reviewed in reference 8). One mechanism by which SAPKs, and MAPKs in general, modulate gene expression is by direct modification of transcription activators. In *Saccharomyces cerevisiae*, only four transcription factors, Sko1, Hot1, and the redundant Msn2 and Msn4, have been proposed to be controlled by the Hog1 MAPK. These factors are unrelated, and the mechanisms by which Hog1

regulates their function may differ from one to another. Hot1, Msn2, and Msn4 activate transcription, whereas Sko1 represses and activates different subsets of osmotic-inducible and Hog1-regulated genes (15, 17, 18). Sko1 is an ATF/CREB factor that represses genes under nonstress conditions by the recruitment of the general corepressor complex Cyc8-Tup1. In response to osmotic stress, Sko1 is phosphorylated by Hog1, thereby relieving repression (15, 16). Msn2 and Msn4 are generic stress factors controlled by PKA and Hog1 by an unknown mechanism. Hot1 physically interacts with Hog1, and its binding to DNA and subsequent transactivation activity are regulated by Hog1 kinase activity (1, 19). Global gene expression analyses carried out to dissect the specific roles for each transcription factor have revealed that each of these factors can account for a limited effect on global gene expression by regulation of a small subset of the osmostress-inducible genes (3, 17). Evaluation of the subset of osmostress-responsive genes controlled by each factor revealed that the activators reported to be under the control of Hog1 were not sufficient to explain the impact of Hog1 on gene expression.

The mechanism by which Hog1 regulates gene expression is not completely understood. In particular, there remained the possibility that additional transcription factors were required for gene expression upon stress. Therefore, we undertook a genetic screen to identify new activators under the control of the MAPK. Thus, we found that Smp1, a member of the MEF2C family of transcription factors, is involved in Hog1 signaling. Interestingly, it was reported previously that in metazoan cells, the MEF2C family of transcription factors can be targeted by the mammalian p38 MAPK (11). We show here

* Corresponding author. Mailing address: Cell Signaling Unit, Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra (UPF), C/Doctor Aiguader 80, Barcelona E-08003, Spain. Phone: 34-93-542 2848. Fax: 34-93-542 2802. E-mail: francesc.posas@cexs.upf.es.

that Hog1 and Smp1 interact in vivo, and we have identified the relevant Hog1 phosphorylation sites in Smp1 and analyzed their effect on stress-regulated gene expression in vivo. Furthermore, we report that Smp1 may play an important role not only in osmotic stress responses, but also in a new function for the Hog1 MAPK required for cell survival in the stationary phase.

MATERIALS AND METHODS

Yeast strains. The following yeast strains were used: L40 (*MATa trp1 leu2 his3 LYS2::lexA-HIS3 URA3::lexA-lacZ*), TM141 (*MATa ura3 leu2 trp1 his3*), TM233 (*MAT α ura3 leu2 trp1 his3 lys2 hog1::TRP1*), YEN44 (*MATa ura3 leu2 trp1 his3 smp1::HIS3*), YEN46 (*MAT α ura3 leu2 trp1 his3 lys2 hog1::TRP1 smp1::HIS3*), and YEN114 (*MATa ura3 leu2 trp1 HOG1-HA \times 6-HIS3*). Integration of the *STL1-LacZ* reporter construct (PEN05) in wild-type, *hog1 Δ* , and *smp1 Δ* strains yielded YEN2 (*MATa leu2 trp1 his3 STL1-LacZ URA3*), YEN7 (*MAT α leu2 trp1 his3 lys2 hog1::TRP1 STL1-LacZ URA3*), and YEN48 (*MATa leu2 trp1 his3 smp1::HIS3 STL1-LacZ URA3*). Genomic disruptions were made by long flanking homology PCR-based gene disruption.

Buffers and media. Buffer A consists of 50 mM Tris-HCl (pH 7.5), 15 mM EDTA, 15 mM EGTA, 2 mM dithiothreitol (DTT), 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamide, 5 μ g of pepstatin per ml, and 5 μ g of leupeptin per ml. Alkaline phosphatase buffer consists of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 10 mM MgCl₂. Buffer B consists of 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 2 mM DTT, 1% Triton X-100, 1 mM PMSF, 1 mM benzamide, and 5 μ g of leupeptin per ml. Kinase buffer consists of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, and 2 mM DTT. Phosphatase inhibitor mixture contains 10 mM NaF, 1 mM sodium pyrophosphate, and 10 mM β -glycerophosphate. Sodium dodecyl sulfate (SDS) loading buffer consists of 50 mM Tris-HCl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol. Yeast extract-peptone-dextrose (YPD) medium contains, per liter, 10 g of yeast extract, 20 g of peptone, and 20 g of dextrose. Selective medium contains 1.7 g of yeast nitrogen base (Difco) per liter, 5 g of (NH₄)₂SO₄ per liter, 20 g of dextrose per liter, and supplements (100 mg each of the amino acid[s] uracil or adenine, as appropriate, except where indicated). X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) solid medium contains selective medium buffered with MES [2-(N-Morpholino)ethanesulfonic acid] at pH 7 plus 0.1 mg of X-Gal per ml. All yeast growth was at 30°C.

Plasmids. The *STL1::LacZ* reporter construct PEN05 was generated by cloning the *STL1* promoter (base pairs -824 to +4) by PCR into YIp358R (*CEN URA3*) (12). Plasmid PEN45 carries a 3.4-kbp *EcoRI/SalI* fragment containing *SMP1* in a multicopy vector YEplac181. p426GAG1 (*P_{GAGI}-GST URA3⁺* 2 μ m) is a gift from M. Takekawa (unpublished). The *P_{GAGI}-GST* allows the expression of GST-fusion proteins via the yeast *P_{GALI}* promoter. Full-length wild-type and mutant *SMP1* genes were cloned into plasmid p426GAG1. Full-length *SMP1* and *SMP1 Δ MADS* (which contains a 90-amino-acid terminal deletion of the MADS box domain) were cloned into the *SalI* site of the pBTM116 plasmid to fuse them to the LexA binding domain. pACTII-HOG1 was obtained by fusion of the full-length *HOG1* PCR product with the GAL4 activation domain in pACTII. The bacterial expression plasmid pGEX-4T (Pharmacia) allows the expression of GST-tagged proteins in *Escherichia coli*. Full-length wild-type and several truncated *SMP1* alleles were cloned into the *EcoRI* site of the pGEX-4T plasmid by PCR. Construction of *SMP1* site-directed mutants was carried out by PCR-directed mutagenesis. Each mutation was verified by DNA sequencing. The *SMP1-m4* was sequenced to verify that it contained only the desired mutations. The m1 mutant contains a double amino acid substitution, namely, Ser365 and Ser376 to Ala, while m2 contains the Ser348 and the Ser357 amino acids mutated to Ala. The m4 contains a quadruple amino acid substitution, namely, Ser348, Ser357, Thr365 and Ser376 to Ala. Those mutant alleles were cloned into pGEX-4T for bacterial expression and into p426GAG1 (*P_{GAGI}-GST URA3⁺* 2 μ m) for yeast expression. The *SMP1*-GFP plasmid contains the *SMP1* gene fused to the green fluorescent protein gene (*GFP*) in a multicopy plasmid pRS426.

Isolation of *STL1::LacZ* reporter activators. Wild-type yeast strain TM141 carrying an integrated *STL1::LacZ* reporter (PEN05) was transformed with a yeast genomic library in the multicopy plasmid YEpl13. Colonies were grown in synthetic media containing X-Gal for 5 to 6 days. From approximately 30,000 colonies, 20 positive clones were selected by their ability to induce *STL1* and thus to produce beta-galactosidase. Plasmids were isolated, and their *HOG1* dependence was evaluated in a *hog1 Δ* strain containing the same reporter construct. Plasmids from *HOG1*-dependent positives were isolated and partially sequenced. Candidate genes were subcloned and assayed as before.

Two-hybrid analysis. The two-hybrid analysis was carried out essentially as described previously (5) by using pACTII and pBTM116 as the activation domain plasmid and LexA as the DNA-binding domain plasmid. The plasmids LexA-SMP1 and LexA- Δ MADSMP1 were cotransformed with pACT-HOG1 using the L40 reporter strain. Positive clones were selected and further tested for β -galactosidase activity as follows. Cells ($\sim 5 \times 10^6$) were spotted onto YPD plates, incubated for 5 h at 30°C, and replicated onto nitrocellulose membranes. β -Galactosidase activity was visualized in situ by using X-Gal as described elsewhere (5).

In vivo coprecipitation assays. In vivo interaction of GST-SMP1 with Hog1 was determined by GST pull-down experiments. Mid-log-phase cells were grown in the presence of 2% galactose for 4 h and subjected or not subjected to a brief osmotic shock (0.4 M NaCl, 10 min). Yeast extract (750 μ g, in a mixture of buffer A plus 150 mM NaCl plus phosphatase inhibitor) was incubated with 50 μ l of glutathione-Sepharose beads overnight at 4°C. The beads were washed extensively with buffer A plus 150 mM NaCl, resuspended in loading buffer, and separated by SDS-polyacrylamide gel electrophoresis (PAGE). Immunoblotting was done by using anti-HA monoclonal antibody 12CA5 (Roche) and anti-GST monoclonal antibody (Pharmacia) together with ECL reagent (Pharmacia).

In vivo SMP1 phosphorylation assays. Wild-type and mutant GST-SMP1 proteins were purified as described above but in the presence of buffer A without EGTA and EDTA. Bead-bound proteins were incubated with 40 U of calf-intestinal alkaline phosphatase (Roche) in 50 μ l of alkaline phosphatase buffer for 60 min at 30°C. After alkaline phosphatase treatment, the beads were washed with several column volumes and Smp1 was detected by immunoblotting by using an anti-GST monoclonal antibody.

Purification of GST proteins in *E. coli* and in vitro kinase assays. GST fusion proteins encoding Pbs2(Ee), Hog1, and full-length or truncated Smp1 were constructed by using pGEX-4T (Pharmacia), expressed in *E. coli* DH5, and purified by using glutathione-Sepharose beads (Pharmacia) in buffer B as described previously (14). Phosphorylation of Smp1 by Hog1 was monitored by the following in vitro kinase assay. One microgram of recombinant GST-HOG1 from *E. coli* was activated by phosphorylation by using 0.5 μ g of GST-PBS2(Ee) in the presence of kinase buffer and ATP. After 15 min at 30°C, 5 μ g of wild-type or mutant versions of Smp1, purified from *E. coli*, was added to the previous mixture together with [γ -³²P]ATP (0.2 μ Ci/ μ l). The mixture was then incubated for 5 min at 30°C, and the reactions were terminated by the addition of 2 \times SDS loading buffer. The labeled proteins were resolved by SDS-PAGE and detected by autoradiography.

β -Galactosidase assays. The transformed yeast strains were grown selectively until mid-log phase in the appropriate selective liquid media and then diluted in YPD for 3 h. Logarithmically growing cells (optical density at 660 nm, 0.5 to 0.8) were treated or not treated with 0.4 M of NaCl for 35 min and permeabilized by ethanol-toluene treatment, and β -galactosidase activity was determined as described previously (6).

Cell viability assays. Cell viability was scored essentially as described previously (7). Yeast cells were grown in YPD or minimal medium to an optical density of 0.8 and subjected or not subjected to an osmotic shock (1 M NaCl for 60 min). Viability was determined by adding propidium iodide (PI) at 0.004 mg/ml and measuring the number of PI-positive cells by using a FACSscan flow cytometer (Becton Dickinson, San Jose, Calif.). Cell viability was also determined by adding Phloxin B at 0.5 mg/ml to the untreated or treated cells as before and counting the stained cells by visual microscopy.

GFP fluorescence microscopy. GFP was visualized without fixation by using a Nikon E-600 with an ORCA II CCD camera (Hamamatsu). Images were taken at 100 \times magnification and converted to Photoshop format version 4.0 (Adobe Systems).

RESULTS

The MEF2C-related Smp1 transcription factor regulates HOG-dependent gene expression. Osmotic stress gene expression is strongly defective in *hog1 Δ* cells (see above); however, deletion of the transcription factors described as being under the control of the MAPK has only a limited effect on osmotic stress gene expression. Thus, it was predicted that other transcription factors must exist under the control of the Hog1 MAPK. To identify new Hog1-regulated transcription factors, we conducted a screening by which we isolated genes whose overexpression was able to induce *STL1* expression. The *STL1* gene

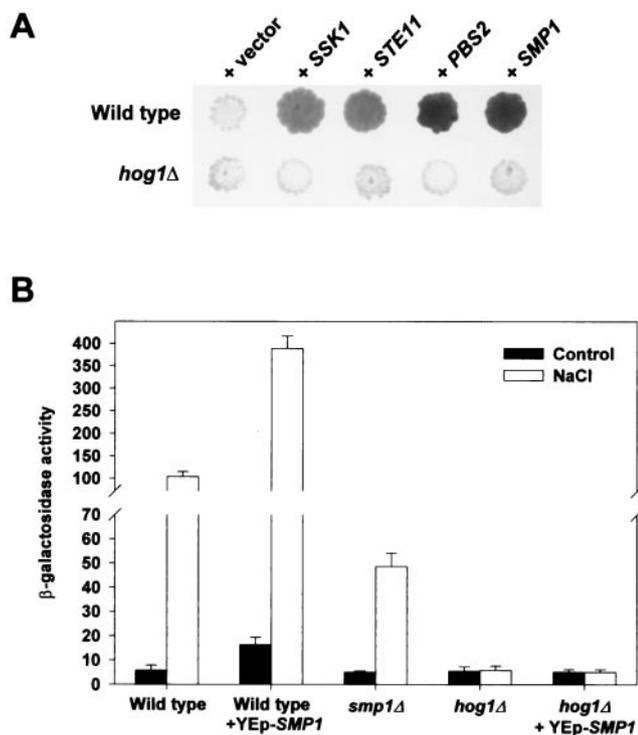


FIG. 1. Smp1 transcription factor modulates *HOG1*-mediated *STL1* gene expression. (A) Yeast multicopy plasmids capable of inducing *STL1-LacZ* expression in wild-type cells were isolated, and their dependence on *HOG1* was assayed in wild-type cells (YEN2) and *hog1Δ* cells (YEN7). A representative filter β -galactosidase assay demonstrating induction of *STL1-LacZ* by several positive clones from the screening is shown. (B) Smp1 modulates expression of *STL1-LacZ*. Wild-type, *hog1Δ*, and *smp1Δ* strains containing the *STL1-LacZ* reporter system (strains YEN2, YEN7, and YEN48) were transformed with a control plasmid or a multicopy plasmid expressing *SMP1*. β -Galactosidase activity was assayed in cells that were grown to mid-log phase and that were subjected (open bars) or not subjected (control; filled bars) to hyperosmotic stress (0.4 M NaCl for 35 min). β -Galactosidase activity is given in nanomoles per minute per milligram and is the result of the measurement in quadruplicate of results for two independent transformants.

encodes a putative sugar transporter, and it is one of the genes that is most strongly induced in response to osmotic stress and is completely under the control of Hog1 (13).

Yeast cells containing an integrated *STL1-LacZ* reporter construct were transformed with a multicopy genomic library, and positive clones were selected by their ability to induce *STL1* and, therefore, to produce β -galactosidase on X-Gal-containing plates (described in Material and Methods). Thus, 20 positive clones were identified from approximately 30,000 colonies. Plasmids were isolated from positive clones and tested again for their ability to induce *STL1* in both wild-type and *hog1Δ* strains containing the *STL1-LacZ* reporter. Only clones that induced *STL1* in a *HOG1*-dependent manner were partially sequenced. From the genomic clones, candidate genes were subcloned into a multicopy plasmid and tested as above.

As expected, some of the clones capable of inducing our reporter system encoded upstream components of the HOG pathway, namely *STE11*, *PBS2*, and *SSK1*, that are known to be able to induce activation of Hog1 by overexpression (Fig. 1A).

In addition to those upstream components of the HOG pathway, we identified elements that could be acting at the level of transcription regulation. We thus identified some genes encoding components of the basic transcription machinery, such as *SRB9* and *SRB4* (data not shown) and a gene encoding the Smp1 protein, a member of the MEF2 transcription factor family (Fig. 1A).

To determine the importance of *SMP1* in regulation of gene expression, we quantified *STL1* expression in liquid β -galactosidase assays. As shown in Fig. 1B, cells overexpressing *SMP1* induced expression of the *STL1* reporter gene to approximately fourfold-higher levels than did wild-type cells under both nonstress and stress conditions. Overexpression of *SMP1* in *hog1Δ* cells did not have any effect on *STL1* expression. Furthermore, deletion of *SMP1* resulted in reduced *STL1* expression (Fig. 1B). Similar results were obtained when *STL1* expression was analyzed by Northern blotting (data not shown). Deletion of the gene that encodes for the Hot1 transcription factor had an even more extensive impact on *STL1* expression than did deletion of the *SMP1* gene, indicating that Hot1 plays a major role in the regulation of *STL1* (data not shown).

The role of Smp1 on gene expression was also assayed by analyzing the expression of other stress-responsive genes known to be controlled by the Hog1 MAPK (13, 17). We created reporter genes containing the *CWP1*, *ALD3*, and *HXT1* gene promoters fused to the LacZ reporter system. Similar to the observations with *STL1*, expression of *CWP1* was induced by overexpression of *SMP1*, whereas deletion of *SMP1* partially reduced its expression upon stress. No *SMP1*-dependent differences on gene expression were observed when the *HXT1* and *ALD3* gene reporters were used, although these reporters were induced under osmotic stress in a *HOG1*-dependent manner (data not shown). Thus, the genetic screen yielded a clone, *SMP1*, encoding a transcription factor of the MEF2 family, capable of regulating a subset of stress-responsive genes under the control of the Hog1 MAPK.

Smp1 is phosphorylated after osmotic stress in a Hog1-dependent manner. When yeast cells are exposed to osmotic stress, the Hog1 MAPK is rapidly phosphorylated and activated (10) (Fig. 2B). To determine whether Smp1 is regulated in response to osmotic stress, we tested whether Smp1 was also phosphorylated upon osmotic stress. Wild-type and *hog1Δ* strains were subjected to a brief osmotic shock, and GST-tagged Smp1 protein was monitored by Western blotting by using anti-GST antibodies. The GST-Smp1 fusion protein was fully functional as tested for its ability to induce *STL1-LacZ* expression (data not shown). As shown in Fig. 2A, when subjected to osmotic stress, the mobility pattern of Smp1 was altered. The mobility change of Smp1 was induced by phosphorylation, because when extracts from osmotic-stressed cells were treated with alkaline phosphatase, the mobility pattern could be reversed (Fig. 2A). Interestingly, Smp1 phosphorylation was rapidly induced upon stress but the kinetics lagged behind those observed for the phosphorylation of Hog1. This kinetic relationship is consistent with Smp1 being dependent on Hog1 (Fig. 2B). Moreover, phosphorylation of Smp1 in response to osmotic stress depends on Hog1, since Smp1 from *hog1Δ* cells did not undergo the mobility shift observed in wild-type cells. Thus, these results showed that Smp1 is rapidly

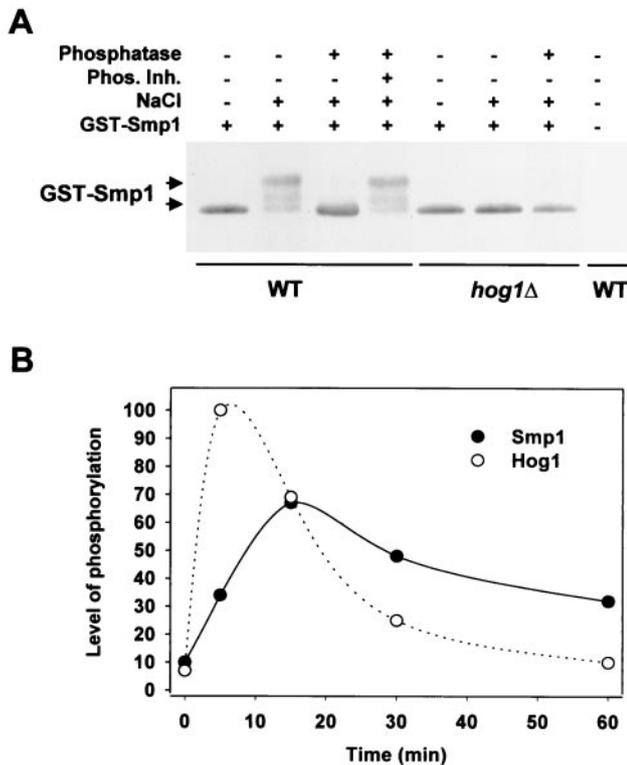


FIG. 2. In vivo phosphorylation of Smp1 upon osmotic stress depends on Hog1. (A) GST-SMP1 was expressed under the P_{GAL1} promoter in wild-type (TM141) and *hog1*Δ (TM233) cells. Yeast cells were grown in the presence of galactose for 4 h and subjected (+) or not subjected (-) to a brief osmotic shock (0.4 M NaCl for 10 min), and the extracts were treated (+) or not treated (-) with 10 U of alkaline phosphatase or phosphatase inhibitors (Phos. Inh.). Extracts were separated by SDS-PAGE, and GST-tagged Smp1 was detected by the use of polyclonal GST-specific antibodies. The control strain (without GST-SMP1) was the wild-type strain (TM141). (B) Time course of Hog1 and Smp1 phosphorylation upon osmotic stress. Cells expressing GST-SMP1 in a wild-type strain were subjected to osmotic stress (0.4 M NaCl), and cells were collected at different times. Phosphorylation of Smp1 and Hog1 was monitored by Western blotting by using anti-GST antibodies and anti-phospho-p38 MAPK (Thr180/Tyr182) antibodies (New England BioLabs), respectively. The level of phosphorylation was measured from scanned films by using Quantity One software (Bio-Rad).

phosphorylated upon hyperosmotic shock in vivo and that this modification depends on the Hog1 MAPK.

Hog1 interacts with the putative transcription activator domain of Smp1. To obtain direct evidence for the interaction of Smp1 and Hog1, we tested by two-hybrid analysis whether these proteins interacted. Smp1 or a mutant containing a deletion on the MADS box and MEF2 domains (amino acids 1 to 90) were fused to the LexA-DNA binding domain, and their interaction with the full-length Hog1 fused to the GAL4 activator domain was tested. Figure 3A shows a typical result and indicates that Smp1 interacts with Hog1 through its C-terminal domain and not through the MADS and MEF2 domains.

The interaction between Hog1 and Smp1, shown by the two-hybrid data, was confirmed by in vivo coprecipitation experiments. Yeast YEN114 cells (which express HA-tagged Hog1 from their own genomic locus) were transformed with a

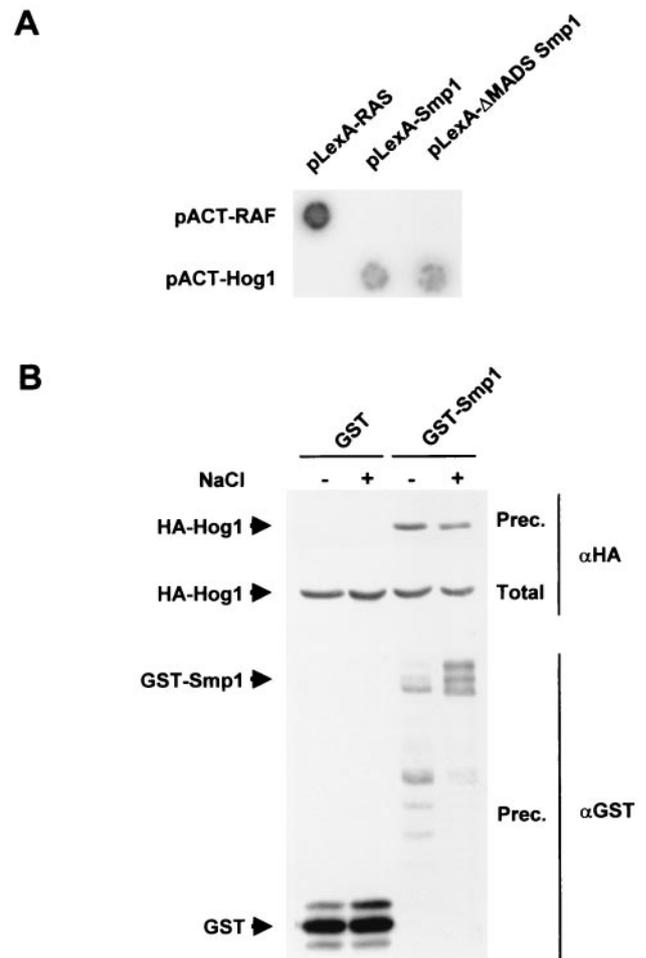


FIG. 3. In vivo binding of Hog1 to Smp1. (A) Interaction as shown by two-hybrid analysis of Hog1 and Smp1. The wild-type version and a mutant that lacks the MADS and MEF2 domains (Δ MADS Smp1) fused to the LexA DB were expressed with the full-length *HOG1* fused to the GAL4 activator domain. A representative filter β -galactosidase assay demonstrating interactions between Hog1 and Smp1 is shown. Proteins encoded by the control plasmids pLexA-RAS^{V12} and pACT-RAF, which are known to interact with each other, are shown for comparison. (B) Smp1 coprecipitates with Hog1. Strain YEN114 (which expresses HA-tagged Hog1 from the wild-type locus) was transformed with a plasmid expressing GST or GST-SMP1 under the P_{GAL1} promoter. Cells were grown in the presence of galactose, and samples were taken before (-) or 10 min after (+) the addition of NaCl to a final concentration of 0.4 M. GST proteins were affinity purified through a glutathione-Sepharose matrix; the presence of HA-Hog1 in the precipitates was probed by immunoblotting with anti-HA (indicated by α HA at right), and GST-containing proteins (indicated by α GST at right) were detected by using antibodies against GST. Total, <10% of the input protein; Prec., total amount of Hog1 or GST precipitated.

plasmid that expresses a GST-tagged Smp1 from the *GAL1* promoter. Cells were subjected to a brief osmotic shock, and Smp1 was precipitated by using glutathione-Sepharose beads. The presence of Hog1 in the precipitates was probed with an anti-HA monoclonal antibody. As shown in Fig. 3B, Smp1 was able to coprecipitate Hog1 irrespective of the environmental conditions. Thus, these in vivo binding assays confirmed the conclusion of the two-hybrid analysis indicating that Hog1 binds to the Smp1 transcription factor.

Phosphorylation of multiples sites at the C terminus of Smp1 by Hog1 affects Smp1 function. Smp1 is phosphorylated upon osmotic stress in a Hog1-dependent manner and interacts physically with the MAPK. We then tested whether Smp1 phosphorylation was carried out directly by the Hog1 MAPK by using purified proteins in an *in vitro* kinase assay. For this purpose, Hog1 and a constitutively activated version of Pbs2 [PBS2(EE)] were purified as GST-fusion proteins from *E. coli*. In the first step of the reaction, Hog1 was activated by phosphorylation in the presence of PBS2(EE) and ATP (2). Then Smp1, purified from *E. coli* as a GST-tagged protein, and [γ - 32 P]ATP were added to the reaction. As shown in Fig. 4A, lane 1, full-length Smp1 was phosphorylated by Hog1 (see the asterisk), whereas when a catalytically impaired Hog1 (hog1KN) was used instead of the wild-type Hog1, no phosphorylation of Smp1 was observed (data not shown), thus suggesting that Smp1 is a direct substrate for the MAPK Hog1.

To map the phosphorylation site(s) for Hog1 in Smp1, we created several truncated *SMP1* alleles and expressed them as GST-tagged proteins in *E. coli*. After purification, the same amounts of the pure proteins were subjected to *in vitro* phosphorylation by activated Hog1 (as described above). A truncated form of Smp1 containing the N-terminal region (amino acids 1 to 274) was unable to be phosphorylated by Hog1, whereas a C-terminal region (amino acids 275 to 452) was strongly phosphorylated by the MAPK (Fig. 4, lanes 2 and 3). Thus, phosphorylation of Smp1 by Hog1 occurs in a region which is coincident with the Hog1 binding domain. It is worth noting that when both N-terminal and C-terminal fragments were present in the same assay, the amount of phosphorylation of the C-terminal fragment was strongly reduced (Fig. 4, lane 4).

To further determine the phosphorylation sites in Smp1, we created several truncated versions of the C-terminal domain (amino acids 275 to 452) and expressed them as GST-tagged proteins in *E. coli*. Truncated forms were purified and assayed as described previously. A 30-residue C-terminal deletion had little effect on Smp1 phosphorylation, whereas deletion of the last 90 residues had a stronger effect on phosphorylation (Fig. 4, lanes 6 and 7). Four sequences corresponding to the consensus phosphorylation site for MAPKs (Ser-Pro/Thr-Pro) are present in the region comprised of residues 275 to 423. We created point mutant versions to replace Ser and Thr residues with Ala, and we tested them for phosphorylation by Hog1. Single mutations of Ser 348, Ser 357, Thr 365, and Ser 376 did not alter Smp1 phosphorylation (data not shown). We then constructed several combinations of mutant sites and tested them by phosphorylation. Mutation of both Thr 365 and Ser 376 (m1) did not change the level of phosphorylation, whereas mutation on both Ser 348 and Ser 357 (m2) slightly decreased the phosphorylation of Smp1 (Fig. 4B). Smp1 phosphorylation was only abolished in the Smp1-m4 mutant, which contains mutations in all four sites (Fig. 4B).

In vivo phosphorylation assays carried out as described previously in wild-type cells transformed with a wild-type GST-tagged Smp1 or the mutant Smp1-m4 protein demonstrated that mutation of the four sites in the Smp1-m4 protein eliminated most of the mobility shift due to phosphorylation of Smp1 in response to osmotic stress (Fig. 5A). Taken together, these results indicate that there is a specific cluster of four

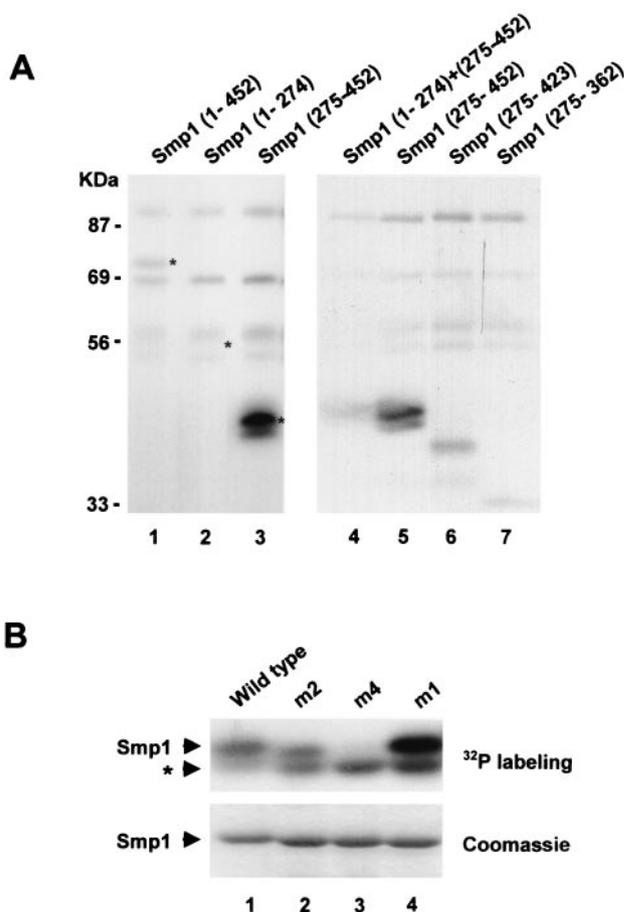


FIG. 4. Hog1 phosphorylates the C-terminal domain of Smp1. (A) *In vitro*-activated Hog1 phosphorylates Smp1. Various Smp1 fragments were tested for their ability to be phosphorylated by an *in vitro*-activated Hog1. The positions of the Smp1 fragments included in the constructs are indicated in parentheses. Recombinant tagged proteins were purified from *E. coli* and subjected to phosphorylation by activated Hog1 as described in Materials and Methods. Phosphorylated proteins were resolved by SDS-PAGE and detected by autoradiography. Asterisks indicate the positions of Smp1 proteins detected by Coomassie staining. (B) Mutation of Smp1 Ser348, Ser357, Thr365, and Ser376 to Ala abolishes Hog1 phosphorylation. The wild-type Smp1 fragment (amino acids 275 to 452) and various Smp1 site-directed mutants were tested for Hog1 phosphorylation as described in the legend for panel A. The Smp1 mutants and their corresponding mutations (indicated in parentheses) are as follows: m2 (Ser348 and Ser357 to Ala), m4 (Ser348, Ser357, Thr365, and Ser376 to Ala), and m1 (Thr365 and Ser376 to Ala). After phosphorylation, the proteins were resolved by SDS-PAGE, and phosphorylated proteins were detected by autoradiography (upper panel). GST-tagged Smp1 proteins were detected by Coomassie blue stain (lower panel). The asterisk indicates a contaminant phosphorylation not corresponding to Smp1.

phosphorylation sites for Hog1 that is located within 28 amino acids in the C terminus of Smp1.

To assess the role of Hog1 phosphorylation in Smp1, we studied the effect of the mutation of the phosphorylation sites in gene expression. Wild-type and *smp1Δ* cells were transformed with a vector containing full-length *SMP1* or the *SMP1-m4* mutant allele (unphosphorylatable by Hog1) under the wild-type promoter, and the effect on transcription was measured by expression of the *STL1::LacZ* reporter assay as

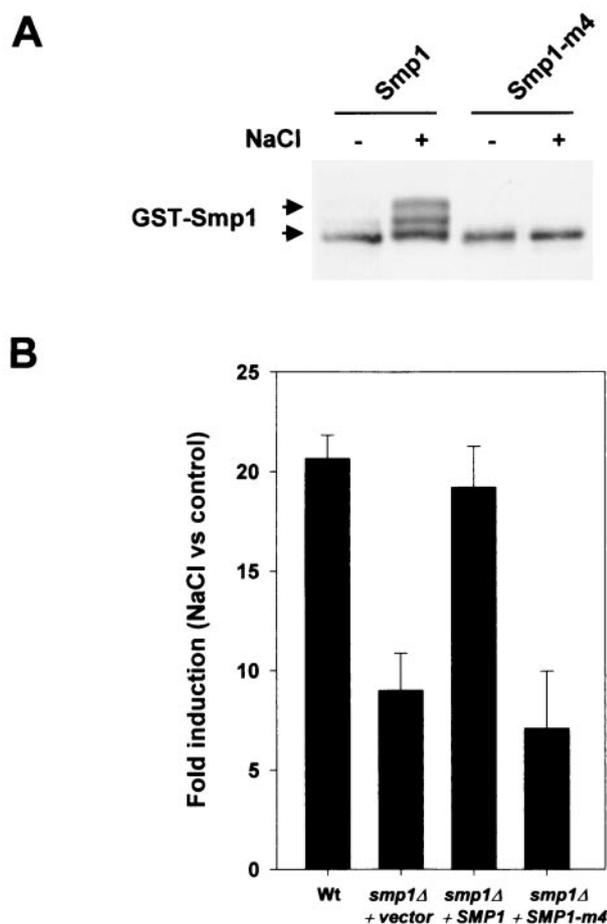


FIG. 5. Smp1 phosphorylations are required for Smp1 transcriptional activity. (A) Mutation of Ser348, Ser357, Thr365, and Ser376 to Ala abolishes in vivo Hog1 phosphorylation. Full-length Smp1 and the quadruple-mutant protein Smp1-m4 (mutations: Ser348, Ser357, Thr365, and Ser376 to Ala) were tested for Hog1 phosphorylation as described in the legend for Fig. 2A. After phosphorylation, the proteins were resolved by SDS-PAGE and detected by immunoblotting by using anti-GST polyclonal antibodies. (B) Hog1 phosphorylation modulates Smp1 function. A wild-type strain and the *smp1Δ* mutant strain (YEN44) containing the reporter *STL1::lacZ* were transformed with a centromeric plasmid expressing wild-type *SMP1* or the quadruple-mutant *SMP1-m4* (unphosphorylatable by Hog1). The cells were subjected to a brief osmotic shock, and β -galactosidase activity was assayed as described in the legend for Fig. 1B. β -Galactosidase activity is given as fold induction of control versus that of NaCl-treated cells and is the result of the measurement in quadruplicate of results for two independent transformants.

before. As shown previously, deletion of *SMP1* resulted in a decrease of *STL1* expression upon osmotic stress. Expression of wild-type *SMP1* restored the levels of *STL1* expression to wild-type levels upon stress, whereas expression of the *SMP1-m4*, which is no longer phosphorylatable by Hog1, was completely unable to restore *STL1* expression in *smp1Δ* cells (Fig. 5B). Smp1 alleles carrying simultaneous mutations of Ser 348 and Ser 357 (m2) or Thr 365 and Ser 376 altered expression of *STL1* only to a minor extent (*STL1* expression was 76 and 90% of that of the wild-type allele, respectively). These results indicated that the Hog1 phosphorylation plays an important role in the regulation of Smp1 transcriptional activity.

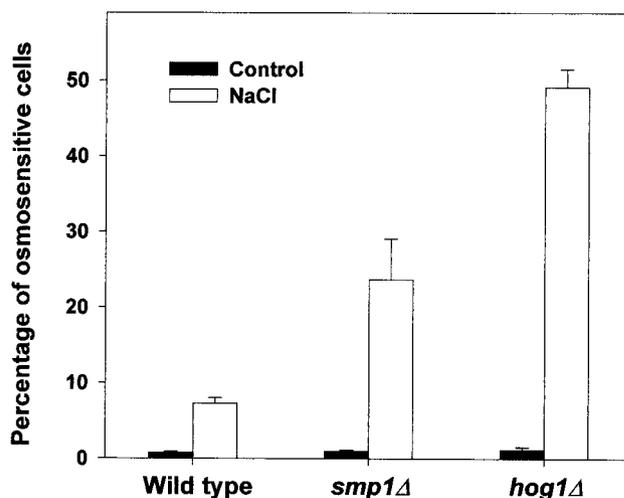


FIG. 6. Cell survival upon osmotic stress is reduced in *hog1Δ* and *smp1Δ* strains. To analyze the role of Smp1 in osmotic stress adaptation, we measured the incorporation of PI, as described in Materials and Methods, as a measure of cell viability in wild-type, *hog1Δ* mutant, and *smp1Δ* mutant strains. Yeast cells were grown in YPD and subjected (open bars) or not subjected (filled bars) to an osmotic shock (1 M NaCl for 60 min). Viability was determined by the addition of PI and measurement of the percentage of PI-positive cells by using a flow cytometer. The results are the measurements in duplicate from six independent experiments. The data were confirmed by Phloxin B staining and visual microscopy (see Materials and Methods).

Smp1 controls part of the Hog1-mediated osmotic stress responses. Smp1 regulates the expression of several stress-response genes. To understand the role of Smp1 in osmotic stress adaptation, we aimed to determine the importance of the presence of Smp1 to the generation of osmotic stress responses. Deletion of *SMP1* did not cause an obvious osmosensitivity phenotype on plates containing sorbitol or NaCl (data not shown). This was similar to the results observed with cells containing deletions of any of the known transcription factors under the control of the MAPK. However, it was previously reported that *hot1*-deficient cells were more sensitive than wild-type cells to a severe osmotic shock (19). Thus, we tested whether *smp1Δ* cells were viable after an osmotic shock. Exponentially growing wild-type, *smp1Δ*, or *hog1Δ* cells were subjected to an osmotic shock (1 M NaCl for 60 min), and viability was determined as described in Materials and Methods. As depicted in Fig. 6, *hog1Δ* cells were highly sensitive to an osmotic shock compared to the wild-type cells. Interestingly, *smp1Δ* cells were also sensitive to an osmotic shock, although less sensitive than *hog1Δ* cells. Similarly to the results observed for the *STL1-LacZ* expression, a wild-type allele of *SMP1* was able to suppress *smp1Δ* osmosensitivity, whereas the *SMP1-m4* mutant allele was unable to restore cell viability. Double-mutant *hog1Δ smp1Δ* cells were as sensitive as *hog1Δ* cells (data not shown). Thus, these data indicated that Smp1 plays a role in the generation of osmotic stress responses, most likely by the regulation of a subset of the osmotic stress-responsive genes controlled by the Hog1 MAPK.

Both Smp1 and Hog1 are relevant for cell viability in stationary phase. To study the mechanism of Smp1 action, we analyzed the cellular distribution of Smp1. For this purpose,

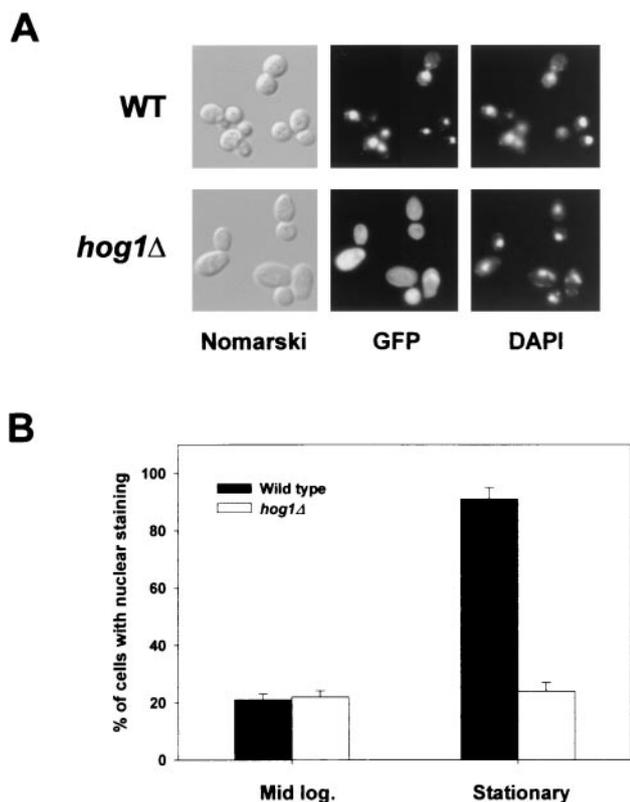


FIG. 7. Smp1 nuclear localization depends on Hog1. The wild-type and the *hog1*Δ mutant strains were transformed with a plasmid carrying a GFP-tagged Smp1 expressed under its own promoter. The cells were grown and Smp1-GFP was detected by fluorescence microscopy as described in Materials and Methods. (A) Smp1 localizes into the nuclei of wild-type cells in the stationary phase. Cells of the indicated types carrying Smp1-GFP were grown to the stationary phase, and images were taken. Representative images showing the localization of Smp1-GFP are presented. The positions of the nuclei were determined by DAPI staining. (B) Quantitative data were obtained from wild-type or *hog1*Δ cells growing at the mid-logarithmic (Mid log.) or stationary phase of growth. The data represent the results of three independent experiments which included counting 300 cells each.

we fused *SMP1* to the N terminus of the GFP and expressed the fusion in yeast under its own promoter. The Smp1-GFP fusion protein was fully functional as tested for *STL1-LacZ* expression (data not shown). Microscopic examination of cells expressing Smp1-GFP revealed that Smp1 localizes throughout the cytoplasm and nuclei of unstressed cells. When cells carrying Smp1-GFP were exposed to a brief osmotic shock (0.4 M NaCl for 5 min), Smp1 did not change its distribution significantly. However, strong nuclear localization was observed in most of the wild-type cells when they were grown in stationary phase (Fig. 7). Nuclear localization of Smp1-GFP was abolished by elimination of Hog1 activity, either by gene deletion or by impairing its catalytic activity (Fig. 7). However, it is worth noting that distribution of the nonphosphorylatable Smp1-m4 protein fused to GFP was similar to that observed in the wild type (data not shown). These results suggested that Hog1 could be playing a role during stationary phase by indirectly controlling Smp1 localization.

It was reported that deletion of the Hog1 homologue Spc1/

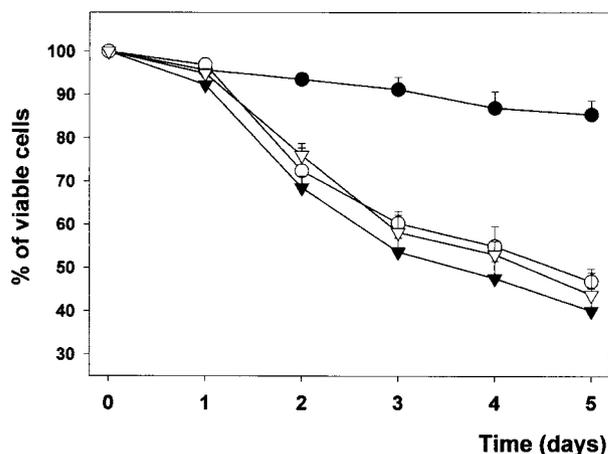


FIG. 8. Hog1 and Smp1 are required for cell viability in stationary phase. Wild-type (●), *hog1*Δ (▽), *smp1*Δ (○), or *hog1*Δ *smp1*Δ (▼) cells were grown in minimal medium to stationary phase and allowed to grow for the indicated period of time. Viability was determined by adding PI and measuring the percentage of PI-positive cells by using a flow cytometer. The results are the measurements in duplicate from four independent experiments. The data were confirmed by Phloxin B staining and visual microscopy (see Materials and Methods).

StyI MAPK from *Schizosaccharomyces pombe* resulted in cells with less viability during the stationary phase (22). To study whether Hog1 and Smp1 were playing a role during this phase of growth, we monitored the cell viabilities of wild-type, *smp1*Δ, and *hog1*Δ cells during 5 days of culture in stationary phase as before. As shown in Fig. 8, wild-type cells maintained cell viability throughout the culture period, whereas *smp1*Δ and *hog1*Δ cells lost viability during growth in the stationary phase. Similar results were obtained with the W303 strain background (data not shown). Furthermore, *smp1*Δ *hog1*Δ double-mutant cells did not show differences compared to *hog1*Δ cells. It is worth noting that whereas a plasmid containing wild-type *SMP1* was able to restore the cell viability of a *smp1*Δ strain, a plasmid containing the *SMP1-m4* allele (encoding the Smp1 version unphosphorylatable by Hog1) was not able to complement *smp1*Δ mutation (data not shown). Thus, these data suggest that apart from controlling osmolarity responses, Smp1 plays an important role for cell survival during growth in the stationary phase and that this role could be mediated by the MAPK through regulation of Smp1 localization.

DISCUSSION

Yeast cells respond to increases in osmolarity in the extracellular environment by activating the stress-activated MAPK Hog1. A major outcome of the activation of the Hog1 MAPK is the regulation of gene expression. One of the most common mechanisms by which SAPKs regulate gene expression is by modification of specific transcription factors (9), and several transcription regulators have been proposed to be controlled by the Hog1 MAPK. However, due to their DNA binding specificities and the profile of gene induction upon stress shown by DNA microarrays, they cannot account for the regulation of all of the genes under the control of Hog1. In

addition, a mutant strain carrying deletions in *hot1*, *msn1*, *msn2*, and *msn4* is not osmosensitive (19).

From these considerations, we suspected that additional transcription factors were required for the osmotic stress-induced regulation of gene expression by the MAPK Hog1. In this report, we describe a genetic screen in which we identified Smp1, a MEF2-like protein, as one such factor. The results of this study demonstrate that both overexpression and deletion of *SMP1* result in altered expression of osmosensitive genes such as *STL1* and *CWP1* but not of others, such as *ALD3* or *HXT1*. It was reported that Smp1 has MEF2-related DNA-binding specificities (4), and a conserved sequence similar to the predicted DNA binding site for Smp1 was consistently present on the promoters of the osmosensitive *STL1* and *CWP1* but not *HXT1* and *ALD3* genes (Fig. 1 and data not shown).

Several lines of evidence suggest that Smp1 is actually a direct substrate for the MAPK. In vivo studies suggested that Hog1 was able to interact with Smp1 and that Smp1 was phosphorylated upon stress in a *HOG1*-dependent manner. The relationship of Smp1 and Hog1 in vivo are further supported by the in vitro evidence that Hog1 phosphorylates Smp1 directly. Four independent phosphorylation sites were mapped within 28 amino acids. Similarly, phosphorylation of Sko1 by Hog1 was restricted to three phosphorylation sites clustered within 19 amino acids (15). The introduction of several phosphates in a small pocket of amino acids might result in conformational changes required for switching the function of those transcriptional regulators. In vitro phosphorylation studies also showed that full-length Smp1 is phosphorylated less efficiently by Hog1 than by the C-terminal domain alone (Fig. 3, lanes 1 and 3). This observation raised the possibility that a region of Smp1 could be limiting the access of the MAPK to the phosphorylation sites. Consistent with this observation was the fact that simultaneous incubation of the C-terminal domain with an N-terminal domain (which is not phosphorylated by Hog1) resulted in a dramatic decrease of C-terminal phosphorylation (Fig. 4, lane 4). This might suggest that Smp1 must be in a preactivated state (i.e., bound to DNA or interacting with other factors) to be accessible for phosphorylation and activation by the MAPK.

Phosphorylation of Smp1 by Hog1 is important for Smp1 function. Upon osmotic stress, Smp1 is strongly phosphorylated, and mutation of the phosphorylation sites to Ala results in an Smp1 that is unable to regulate gene expression (Fig. 5). In yeast, there exists a second MEF2-related protein, Rlm1, that is under the control of the Slr2/Mpk1 MAPK. Interestingly, Mpk1 phosphorylates Rlm1 in a region similar to that found for Smp1, and this phosphorylation results in an increase of its transcriptional activity (4, 23). Thus, two independent MAPK signaling pathways could be controlling the two MEF2-related factors by a similar mechanism. In mammals, regulation of MEF2A and MEF2C factors has been shown to be under the control of the p38 MAPKs, among other kinases (reviewed in references 9 and 11). Phosphorylation of the transcription activator domain of these factors by p38 stimulates MEF2 activity, which is analogous to the mechanism proposed for the yeast Smp1.

Apart from the role of Smp1 in the regulation of a subset of osmosensitive genes under the control of the Hog1 MAPK

(and thus a role in osmoadaptation), we found that *smp1Δ* cells lose viability in the stationary phase, as occurs with *hog1Δ* cells (Fig. 8). This phenotype is reminiscent of that observed upon deletion of the *Spc1/StyI* MAPK in *Schizosaccharomyces pombe* (21, 24), and although the molecular mechanism(s) of this deficiency remains uncharacterized, the formal possibility exists that this could be caused by the lack of some specific transcript required for this phase of growth. As shown in Fig. 7, Smp1 concentrates into the nucleus when cells enter into the stationary phase, and this could be of relevance for the induction of specific genes. In *hog1Δ* cells, Smp1 nuclear accumulation is clearly diminished, and thus this could impede the normal function of Smp1. Because *smp1Δ* and *smp1Δ hog1Δ* cells display similar viability, it is likely that the deficiency observed in *hog1Δ* cells could be caused by the improper function of Smp1 rather than other mechanisms.

Taken together, our results show that Smp1 is a direct target for the Hog1 MAPK and that two different levels of regulation can be controlling the activity of this transcription factor. Under stress, Hog1 phosphorylates the putative transcription activation domain stimulating Smp1 activity, and, when entering into the stationary phase, Hog1 is required for proper localization of the transcription factor.

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The first two authors contributed equally to this work.

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