Ineffective Phosphorylation of Mitogen-Activated Protein Kinase Hog1p in Response to High Osmotic Stress in the Yeast Kluyveromyces lactis

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When treated with a hyperosmotic stimulus, Kluyveromyces lactis cells respond by activating the mitogen-activated protein kinase (MAPK) K. lactis Hog1 (KlHog1) protein via two conserved branches, SLN1 and SHO1. Mutants affected in only one branch can cope with external hyperosmolarity by activating KlHog1p by phosphorylation, except for single ΔKlste11 and ΔKlste50 mutants, which showed high sensitivity to osmotic stress, even though the other branch (SLN1) was intact. Inactivation of both branches by deletion of KISHO1 and KLSSK2 also produced sensitivity to high salt. Interestingly, we have observed that in ΔKlste11 and ΔKlsho1 Δklssk2 mutants, which exhibit sensitivity to hyperosmotic stress, and contrary to what would be expected, KlHog1p becomes phosphorylated. Additionally, in mutants lacking both MAPK kinase enzymes (MAPKKs) present in K. lactis (KlSte11p and KlSsk2p), the hyperosmotic stress induced the phosphorylation and nuclear internalization of KlHog1p, but it failed to induce the transcriptional expression of KISTL1 and the cell was unable to grow in high-osmolarity medium.

KlHog1p phosphorylation via the canonical HOG pathway or in mutants where the SHO1 and SLN1 branches have been inactivated requires not only the presence of KlPbs2p but also its kinase activity. This indicates that when the SHO1 and SLN1 branches are inactivated, high-osmotic-stress conditions activate an independent input that yields active KlPbs2p, which, in turn, renders KlHog1p phosphorylation ineffective. Finally, we found that KlSte11p can alleviate the sensitivity to hyperosmotic stress displayed by a ΔKlsho1 Δklssk2 mutant when it is anchored to the plasma membrane by adding the KlSho1p transmembrane segments, indicating that this chimeric protein can substitute for KlSho1p and KlSsk2p.

Yeasts contain evolutionarily conserved signaling mitogen-activated protein kinase (MAPK) pathways that are utilized under many stressing environmental conditions. One of them is the HOG pathway, which is dedicated to the production of compatible solutes, such as glycerol, needed to cope with external high osmolarity (1). In Saccharomyces cerevisiae, the HOG pathway is composed of two branches, each of which contains a transmembrane protein, Sln1p (2) or Sho1p (3), which acts on downstream proteins. These two branches converge in the MAP kinase kinase (MAPKK) Pbs2p. The scaffold and kinase activities of Pbs2p are required to activate the MAPK Hog1p by phosphorylation, which, once it is active, goes into the nucleus to regulate the transcription of several genes (4, 5).

The SLN1 branch is a phosphorelay system composed of the histidine kinase Sln1p, the phosphotransfer protein Ypd1, and the response regulator Ssk1p. Upon stress stimulation, Sln1 inhibits its autophosphorylation, which renders unphosphorylated Ypd1 and Ssk1 proteins. The last protein triggers the autophosphorylation of the redundant MAPKKks Ssk2p and Ssk22p, the subsequent phosphorylation of Pbs2p, and the activation and nuclear internalization of Hog1p (2, 4).

The other branch, SHO1, is composed of several proteins, some of which are shared with the pheromone response and filamentous MAPK pathways. This branch includes Ste20p, Ste50p, and the MAPKKK Ste11p. Sho1p is a protein that has four transmembrane domains, separated by three short loops of 5 to 8 amino acids each (3). Sho1p predominantly localizes in the plasmatic membrane at areas of polarized growth (6, 7). This protein binds to the p21/activated kinase (PAK) Ste20p, which in turn activates the MAPKKK Ste11p (8). The Sho1 C-terminal cytoplasmic region contains an SH3 domain and binds Pbs2p and the complex of Ste11p and its adaptor protein, Ste50p (3, 9). Plasma membrane anchoring seems to be required for the efficient activation of Pbs2p by Ste11p (8).

The SLN1 and SHO1 branches have largely been defined to be redundant, since both pathways can stimulate the phosphorylation of Pbs2p and the activation and nuclear translocation of Hog1p, allowing growth on high-osmolarity media (10). Consequently, Hog1p activation is prevented by inactivation of Pbs2p and/or when both pathways are inactivated upstream of Pbs2p.

Although the HOG pathway has been very well studied in S. cerevisiae, only a few studies have been done in other yeast species. From those, the best-characterized osmостress pathways are those from Schizosaccharomyces pombe and Candida albicans. In S. pombe, the StyI pathway displays some similarities with the HOG pathway, in that it responds to osmotic stress and controls the

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transcription of genes required for osmoadaptation (11, 12). However, it has been shown that Sty1p phosphorylation can be also stimulated by a variety of stress conditions, including heat shock, oxidative stress (11, 13), and nitrogen and carbon starvation (14, 15). Moreover, the architecture of the Sty1 pathway shows important differences from the HOG pathway. It does not present a Sho1 protein, and Sty1p regulation is controlled by a phosphorylating signaling system which signals oxidative but not osmotic stress (16).

Like S. pombe, the Candida albicans Hog1p (CaHog1p) stress-activated protein kinase pathway also responds to a variety of stress conditions. CaHog1p is activated in response to osmotic and oxidative stresses (17, 18), carbon stress (19), and heavy metals (18), among other stresses. Although C. albicans presents genes homologous to those in the S. cerevisiae SHO1 branch, including the gene for MAPKKK CaSte11p, these proteins may play no role in CaHog1p activation under osmotic stress (20); instead, the osmotic stress response seems to depend on phosphorylase systems highly related to those in S. cerevisiae and S. pombe (12, 21).

Kluyveromyces lactis is an ascomycetous budding yeast closely related to S. cerevisiae that has become an alternative and attractive model since it owns distinct metabolic and physiological properties (22, 23). Comparative genomic analyses have shown that K. lactis contains orthologs of most proteins of the S. cerevisiae HOG pathway (24). However, besides one genetic study (25) and the report that a mutant with a deletion of the gene, encoding a serine-threonine protein kinase involved in the mitotic stress response seems to depend on phosphorelay systems (26). Here we describe some aspects of K. lactis Hog1p regulation, focusing on the role of the SHO1 branch.

**MATERIALS AND METHODS**

**Strains, media, and plasmids.** The Kluyveromyces lactis strains used in this work were MD2/1 (MATa argA lys2 ura3) (27) and 155 (MATa ade2 his3 ura3) (28). The Escherichia coli DH5α strain was used to propagate plasmids and to produce DNA for sequencing. LB medium supplemented with 100 µg/ml ampicillin was used to select recombinant bacteria. Yeast extract–peptone-dextrose (YPD) medium contained 1% yeast extract, 2% Bacto peptone, and 2% glucose. 5-Fluoroorotic acid (5-FOA; 1 mg/ml) was added to YPD medium for negative selection of the URA3 cassette. Synthetic dextrose (SD) medium (0.67% yeast nitrogen base without required amino acids and nitrogen bases to grow yeast strains carrying plasmids. S. cerevisiae strain was used to propagate plasmids. SGal medium was the same as SD medium, except for the replacement of glucose by galactose. Standard protocols were used for plasmid isolation, transformation, and subsequent sequencing of PCR products. The integrative vector YIp352-URA (25) was used for yeast gene disruptions. YeplKDGal (29) was used for galactose induction of gene expression.

**Gene cloning and disruption.** K. lactis ΔKlste20, ΔKlste50, ΔKlste11, ΔKlpbs2, and ΔKlhog1 mutants were constructed by homologous recombination as described before (23) and are isogenic to the MD2/1 strain. KISHO1 and KISSK2 were identified by a BLAST search of the K. lactis genome database Genolevures project II (http://genolevures.org/klla.html). The KISHO1 gene was cloned by PCR amplification using primers Sho1a [(−7) CAGAGAGATGCGGTATCAT ([+11])] and Sho1b [(+1076) ATCAGTGATGTATCCCAT (+1059)]. The PCR product was obtained by PCR using primers Ssk2a [(−19) CAGTGGCGTCCATTAAGGCCA] and Ssk2b [(+676) AACCTTTATGTCCTGAGACT AGTT (+4650)], in which substitutions (lowercase nucleotides) were introduced in order to insert Sall and Xbal restriction sites (underlined), respectively. The PCR products were ligated into the pGEM-T Easy vector. For KISHO1 gene disruption, a BclI-PstI (496-bp) fragment within KISHO1 was replaced by the URA3 sequence. The resulting construct, pGEM-Sho1URA3, was linearized with EcoRI and SacI (leaving 399- and 185-bp flanking ends) and used for gene disruption. For KISSK2 gene disruption, an EcoRI-HindIII (1,861-bp) fragment present within the gene was introduced into the YIp352 vector, which had previously been digested with the same enzymes. The resulting plasmid was digested at positions +1189 and +1505 with Xhol and used for gene disruption. Gene disruptions in MD2/1 mata cells were achieved by homologous recombination, selecting for URA3" cells. Ura" segregants were obtained by plating cells on YPD medium containing 5-FOA until colonies appeared. A ΔKlhog1 ΔKlhsk2 double mutant was constructed by transforming a ΔKlhsk2 Ura" segregant with the fragment carrying KISHO1 and KIURA3 and selecting for URA3" cells. All gene disruptions were confirmed by Southern blotting. The KIPBS2 gene was cloned by PCR amplification using primers Pbs2a [(−7) TCCAAATTGAGTATAG (+11)] and Pbs2b [(+2183) AGCTTAACCAATCCCG (+2166)] and subcloned into pGEM-T Easy. The KLPbs2dup allele (where KM indicates the replacement of Lys 447 by Met) was constructed by PCR using primer (+1327) TAATGGCCATGATGAAAGTCAGATT (+1351) as a forward and mutagenic primer, in which the original adenine at position 1339 was replaced by thymidine (in bold), and primer Pbs2b. A second PCR was done using forward primer Pbs2a and the product of the 1st PCR as the reverse primer. The final product was subcloned into pGEM-T Easy, This renders the replacement of Lys 447 by Met. Both the wild-type and the mutated genes were obtained from pGEM-T Easy as blunt-ended NotI fragments and subcloned into YeplKDGal digested with BamHI and filled in with the Kloewen enzyme.

KISHO1ΔKlste11 fusion. pGEM-Klste11, which was previously described (25), was digested with Xhol, generating a 2,193-bp fragment, which was incubated with the Kloewen enzyme and then ligated into YeplKDGalURA (28), which had previously been digested with EcoRI and filled in with the Kloewen enzyme, yielding YeplKDGal-Klste11. A protein consisting of a fusion between full-length Klste11p and the transmembrane segments of KISHO1 was constructed as follows: a 360-bp fragment of KISHO1 tagged with 6 histidine residues was obtained by PCR using pGEM-KISHO1 as the template. For this we used forward primer (F-Sho1) [(+1) ATGACACACACACACACACACACACTTGGGGAC ATTTTTG (+21)], which contains a translation start codon followed by 6 histidine in-frame codons (in bold) and the hybrid primer R-Sho1/Ste11 (TGACAGCTACTCTCAGTTGGAGATGCAAAACGCCC (+2194)), which contains modifications (lowercase nucleotides) to introduce Xhol restriction site (underlined). The final PCR product was subcloned into pGEM-T Easy and sequenced in full. The KISHO1/Ste11 hybrid gene was obtained as a 2,565-bp NcoI-XhoI fragment from the pGEM-T Easy plasmid, filled in with the Kloewen enzyme, and subcloned into the blunt-ended BamHI YEpKDGalura vector, YeplKDGalSho1(TM), which contains only the four transmembrane (TM) segments of Kloew1p, was constructed by PCR using the F-Sho1 primer and the reverse primer (+340) TGAGATGCTTATCC (+488), which contains a stop codon (underlined). The PCR product was subcloned into pGEM-T Easy. An EcoRI 372-bp fragment from the last construct was subcloned into YeplKDGalURA that had previously been digested with the same enzyme, giving rise to YeplKDGalSho1(TM).

Klhog1-GFP fusion. The Klhog1 gene, including its own promoter was amplified by PCR using the forward primer F-Hog1 [(−499) TTCG
CAAAATCTTACCTGA (−482) and reverse primer R-Hog1 [(+1352) GT TTAATTGATAGCaACATTTT (+1323)], in which substitutions (lowercase nucleotides) were introduced to create a CiaI site before the *KlHOG1* stop codon. The PCR product was subcloned into the pGEM-T Easy plasmid. A 1.834-bp PstI-ClaI fragment was obtained from the pGEM-T Easy clone and was introduced into pSGFP (obtained from Bertha Michel) that had been digested with the same enzymes. This places the *KlHOG1* gene in frame with the gene for the green fluorescent protein (GFP), which yields KlHog1p tagged with GFP in its C terminus. The hybrid gene was obtained from pSGFP as a PstI-HindIII fragment and was ligated into YEpkD (28) that had been digested with the same enzymes. This construct, YEpkD-Hog1-GFP, was used for yeast expression.

**Hyperosmotic stress assays.** All strains were grown overnight at 30°C on SD medium supplemented with the required amino acids. Cells were diluted and allowed to grow until the optical density (OD) at 600 nm (OD<sub>600</sub>) in the same medium was 0.4. When required, SD medium was replaced by SGal medium to induce Gal1 promoter expression. To monitor cell growth, exponentially growing cultures were 10-fold serially diluted and spotted onto YPD plates or (SGal plates for strains carrying plasmids) containing 0.7 M KCl or 1 M sorbitol. The plates were incubated for 48 h at 30°C, and photographed. For KlHog1p phosphorylation assays, cells were grown until the OD<sub>600</sub> was 0.4, collected, washed with water, and suspended in fresh medium. Cell suspensions were not treated or treated with 0.5 M KCl for 5 min prior to protein extraction and Western blot assays.

**Mating assays.** A patch of cells of the strain to be tested was grown on a plate of selective medium for 24 h. The tester strain was grown as a lawn on a YPD plate for 24 h. Both strains were replica plated onto YPD plates and incubated for 8 h at 30°C, allowing the cells to mate. Diploids were selected on SD medium by replica plating and photographed 48 h later.

**Western blotting.** Cells were grown overnight at 30°C in YPD or SGal medium (for strains carrying plasmids) and adjusted to an OD<sub>600</sub> of 0.4. Samples were taken and incubated with KCl for 5 min. After incubation, the cells were treated with 85% trichloroacetic acid and centrifuged briefly, and the supernatant was removed by aspiration. The resulting pellets were suspended in lysis buffer (250 mM Tris [pH 6.8], 0.5 M dithiothreitol, 10% SDS, 20% glycerol, 0.5% bromophenol blue) that was adjusted to pH 8.8 with 1.5 M Tris. One volume of 0.5 mM glass beads was added, and lysis was carried out by vigorous vortexing for 3 min. After 1 min of centrifugation, the supernatant was transferred to a clean tube and incubated at 95°C for 5 min. Aliquots were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and blotted onto an Immobilon polyvinylidene difluoride membrane (Millipore Corporation). The blots were blocked as indicated by the supplier and incubated overnight with a rabbit monoclonal anti-phospho-p38 antibody (Cell Signaling Technology). The membranes were washed three times (10 min each) with 1× phosphate-buffered saline (PBS)–0.1% Tween 20 at room temperature (RT). The blots were incubated with a secondary anti-rabbit immunoglobulin anti-heraldshard peroxidase-conjugated antibody (Zymed) for 1 h at RT. Dually phosphorylated Hog1 was visualized with the chemiluminescent assay (Millipore Corporation). Stripping of the membranes of all anti-phospho-p38 blots was performed by incubation of the membrane at 55°C for 30 min in 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, and 2% SDS. The membrane was then re-probed with anti-Hog1p antibody (yc-20; Santa Cruz Biotech) by following the same procedure as the one used for anti-phospho-p38 antibody. The blots were probed with anti-His-peroxidase antibodies for detection of the His<sub>10</sub>-tagged protein according to the directions of the supplier (Roche).

**KLS1 expression.** KLS1 expression was examined by probing nylon membranes containing immobilized total RNA from wild-type, *Δklstk2 Δklste11*, and *Δklhog1* strains. Cells were treated with 0.5 M KCl for different times prior to RNA extraction. An NcoI-EcoRI 600-bp fragment from the KLS1 gene, obtained from a pGEM-STL1 clone, was labeled with [α-<sup>32</sup>P]dCTP by use of a Prime-II random primer labeling kit (Stratagene) and used as a probe. The RNA blot was hybridized at 55°C, followed by three 10-min washes at 60°C. KLS1 RNA was detected by autoradiography. RNA was purified by the hot acidic phenol protocol. The standard methodology was used for Northern blot analysis.

**Fluorescence microscopy.** Cells were grown overnight in SD, collected, washed, suspended in SGal medium, and incubated at 30°C for 5 h. Hyperosmotic shock was done with 0.5 M KCl for 10 min, and then the cells were fixed with 37% formaldehyde for 30 min. The cells were pelleted and suspended in 1 ml distilled water. For nucleus staining, fixed cells were treated with 1 mg/ml DAPI (4′,6-diamidino-2-phenylindole) for 30 s. The cells were centrifuged for 1 min and washed three times with 1× PBS. The supernatants were decanted, and the cells were suspended in the residual liquid. Cells were observed under an epifluorescence microscope. Images were taken and adjusted using QCapture Pro (version 6.0) software (QImages).

**RESULTS**

Mutants with deletions of components of the SHO1 and SLN1 branches show variable sensitivity to high osmolarity. To evaluate the *K. lactis* response to high osmolarity, we constructed mutants with deletions of putative components of the SHO1 branch which have been shown to participate in the osmotic response in other species. We included in this analysis the genes that code for *Klhog1*, *Klste11*, MAPKKK *Klste20*, and its adaptor protein, *Klste50*.

As can be seen in Fig. 1A, which shows the results of the spot dilution assay, the growth of the *Δklsho1 Δklste20* mutants was indistinguishable from that of the wild-type strain under conditions of high osmolarity (0.7 M KCl or 1 M sorbitol). However, we observed that KlSte11p or its adaptor protein, KlSte50p, was inactivated, growth was severely affected. All the observed phenotypes are in agreement with those described previously (25), except for that of the *Δklste20* mutant. We attribute this discrepancy...
to the different genetic background in which mutants in this study were constructed. It will be interesting to further investigate the genetic influence of the KISte20p activity on the HOG pathway. The sensitivity of the ΔKlste11 mutant to the high osmolarity caused by sorbitol was almost as strong as the sensitivity shown by mutants where the MAPKK scaffold protein KlPbs2p and the MAPK KlHog1p were eliminated (Fig. 1A). This observation is in contrast to the phenotype displayed by a Δste11 mutant of S. cerevisiae, which showed no sensitivity to high osmolarity; in fact, in this yeast, the osmosensitivity of a Δste11 mutant can be observed only when the SLN1 branch is also inactivated (4). The high sensitivity shown by the ΔKlste11 mutant of K. lactis indicates that this MAPKKK protein may be a point of convergence between the SHO1 and SLN1 branches.

Because of the strong sensitivity to hyperosmotic stress displayed by the ΔKlste11 mutant, we decided to analyze if the SLN1 branch also participates in the response to osmostress. To this end, we disrupted the gene encoding KlSsk2p, which is the only MAKKK present in the SLN1 branch of the HOG pathway in K. lactis. KlSsk2p is the orthologue of Ssk2p from S. cerevisiae. The growth assay with either 0.7 M KCl or 1.0 M sorbitol (Fig. 1B) showed that the ΔKlssk2 mutant is osmosesistant. We then mutated both KISSK2 and KISHO1 to inactivate both K. lactis HOG branches. In contrast to the single ΔKlsho1 and ΔKlssk2 mutants, the double ΔKlsho1 ΔKlssk2 mutant was as sensitive to osmotic stress as the ΔKlhog1 mutant (Fig. 1B), indicating that the K. lactis HOG pathway is, indeed, composed of the two branches working in parallel.

Phosphorylated KlHog1p does not always induce protection against hyperosmotic stress. Analysis of KLHog1p in K. lactis wild-type and mutant strains showed that the total protein level did not change upon osmotic stress (Fig. 2A). Additionally, conditions of high osmolarity induced KLHog1p phosphorylation to wild-type levels in the osmosesistant ΔKlsho1 and ΔKlste20 single mutants (Fig. 2A). Surprisingly, in the ΔKlste11 and ΔKlste50 mutants, which showed high sensitivity to hyperosmotic stress, KLHog1p was also phosphorylated after 5 min of exposure to 0.5 M KCl. The observed KLHog1p phosphorylation occurred only after treatment with high salt concentrations and was not observed when the MAPKK KlPbs2p scaffold protein was absent (Fig. 2A).

KLHog1p phosphorylation in single mutants lacking the SHO1 branch could be caused by the activity of the SLN1 branch. To test this hypothesis, we monitored KLHog1p phosphorylation in cells in which both HOG branches were inactivated. We found that even though the ΔKlsho1 ΔKlssk2 double mutant displayed sensitivity to hyperosmotic stress, KLHog1p was phosphorylated when cells were treated with 0.5 M KCl (Fig. 2B).

Phosphorylation in mutants lacking both HOG branches, which display high sensitivity to osmotic stress, indicates either that KLHog1p phosphorylation is not a prerequisite for activation of a cellular response or that the phosphorylation level of KLHog1p in these mutants is not sufficient to induce protection against hyperosmotic stress. Phosphorylation of Hog1p in mutants lacking both branches of the HOG pathway has been observed in S. cerevisiae cells but only when they are exposed to severe conditions of high osmolarity (30, 31). In contrast, we found that in K. lactis double mutant cells, KLHog1p phosphorylation occurs under conditions with middle levels of osmolarity, as described in this paper.
Since KlHog1p becomes phosphorylated and internalized into the nucleus when cells lacking KlSsk2p and KlSte11p are treated with hyperosmotic stress, we determined whether, under these conditions, KlHog1p is able to induce expression of a target gene. STL1, which encodes a glycerol-proton symporter, is a probed target gene of activated Hog1p in *S. cerevisiae* (32). Upon hyperosmotic shock, STL1 is strongly and transiently expressed in *S. cerevisiae*, and this expression is dependent on Hog1p phosphorylation. We tested if the orthologue homologue KlSTL1 gene can be overexpressed in the wild-type strain and in the Klssk2 Klste11 double mutant. In Northern blot assays, we found that KlSTL1 showed basal levels of expression under isosmotic conditions in both the wild type and the ΔKlsk2 ΔKlste11 double mutant. After a 5-min exposure to high KCl concentration, KlSTL1

**FIG 3** Elimination of KlSsk2p and KlSte11p induces sensitivity to hyperosmotic stress but does not prevent KlHog1p phosphorylation. (A) Effect of high osmolarity on the growth of HOG mutants. Cells of the indicated strains were grown overnight at 30°C in liquid YPD, collected, washed, and diluted in fresh medium. Cells were grown until the OD₆₀₀ was 0.4 and spotted as 10-fold serial dilutions onto YPD plates alone or YPD plates containing 0.7 M KCl. The plates were incubated at 30°C for 48 h and photographed. (B) KlHog1p phosphorylation of MAPKKK single or double mutants. Cells were grown until the OD₆₀₀ was 0.4 and not treated or treated with 0.5 M KCl for 5 min. Cells were lysed, and samples of the resulting extracts were subjected to SDS-PAGE, transferred to nylon membranes, and probed with anti-p38 antibody (P-Hog1p). Membranes were stripped and reprobed with anti-Hog1 antibody. (C) KlHog1p-GFP localization. Cells of wild-type and ΔKlsk2 ΔKlste11 double mutant strains carrying YEpKD352-Hog1-GFP were grown as indicated, not treated or treated with 0.5 M KCl for 5 min, stained with DAPI, fixed, and photographed under an epifluorescence microscope. (D) Overexpression of KlHog1-GFP in wild-type and ΔKlhog1 strains. Cells carrying YEpKD352-Hog1-GFP or vector alone (−) were grown in SD medium until the OD₆₀₀ was 0.4. Cells were collected, washed, suspended in fresh medium, and spotted as 10-fold dilutions onto YPD alone or YPD containing 0.7 M KCl. The plates were incubated at 30°C for 48 h and photographed. (E) Expression of KlSTL1 upon hyperosmotic stress. Wild-type and mutant cells were grown in YPD until the OD₆₀₀ was 0.4, washed, transferred to fresh medium, and treated or not treated with KCl for the indicated times. Total RNA was extracted, subjected to agarose electrophoresis, immobilized on a nylon membrane, and probed with radiolabeled *KlSTL1*. mRNA was detected by autoradiography.
was overexpressed severalfold in the wild-type strain (Fig. 3E). This overexpression was transient and decayed after 15 min. In contrast, KlHog1p failed to activate the transcription of KISTL1 in the Δklssk2 Δklste11 double mutant (Fig. 3E), which strongly suggests that phosphorylated KlHog1p is not functional under these conditions.

Taken together, these results suggest that the phosphorylation of KlHog1p in the double mutant occurs through an independent input of the HOG branches in K. lactis and that this activation is not sufficient to induce the expression of target genes and therefore is unable to produce protection against hyperosmotic stress.

**KlHog1 phosphorylation is dependent on KlPbs2p kinase activity.** Our results indicate that under high osmotic stress, KlHog1p is phosphorylated and accumulates in the nucleus in cells in which both HOG branches have been interrupted. Since this phosphorylation cannot induce protection against hyperosmotic stress, we suggest that a noncanonical pathway is activated in cells in which both HOG branches have been interrupted. Since this phosphorylation cannot induce protection against hyperosmotic stress, we suggest that a noncanonical pathway is activated under these conditions.

**DISCUSSION**

In this work, we demonstrated that K. lactis possess a SHO1 branch dedicated to the control of a response to high osmolarity. Some SHO1 branch components, namely, KlSho1p and KlSte11p, are dispensable, as long as the phosphorylation kinase KISSK1 branch (which also exists in this yeast species) functions normally. Additionally, we have shown that while a Δklsho1 mutant grows normally in high-osmolarity medium, further inactivation of KlSsk2p in this mutant is not sufficient to induce the expression of target genes and therefore is unable to protect cells against high-osmotic stress.

We next asked if KlHog1p can be phosphorylated in a strain deficient in the kinase activity of the MAPKK KlPbs2p. In S. cerevisiae, replacement of lysine 389 by methionine inactivates the kinase activity of Pbs2p, and thus, it cannot be phosphorylated (4) and therefore cannot activate Hog1p. We constructed the equivalent Pbs2p kinase-less allele in K. lactis by replacement of Lys 447 by Met (Klpbs2p[KM]). We monitored the KlHog1p phosphorylation and growth in high-osmolarity medium of cells lacking KlPbs2p and cells overexpressing the Klpbs2p[KM] protein. In these experiments, we did not observe KlHog1p phosphorylation in the ΔKlpbs2p null mutant when it was exposed to 0.5 M KCl (Fig. 4A). Overexpression of the kinase-less version of KlPbs2p in the null mutant did not restore KlHog1p phosphorylation when cells were treated with high-osmolarity medium (Fig. 4A). We conclude that the phosphorylation of KlHog1p is totally dependent on the presence of KlPbs2p and its kinase activity. As expected, elimination of KIPbs2 or inactivation of its kinase activity caused high sensitivity to osmotic stress, as can be seen in the results of the spot dilution assay shown in Fig. 4B.

**KlSte11p may bypass the requirement for KlSsk2p and KlSho1p.** Current models in S. cerevisiae postulate that Ste11p is recruited by Sho1p and this allows its activation by the Ste20p kinase (8, 9). When it is located in those membrane complexes, active Ste11p in turn can activate Pbs2p by phosphorylation, but its activity can be dispensable if the SN1 branch is fully active. Interestingly, mutants of K. lactis lacking KlSte11p show high sensitivity to osmotic stress, even in the presence of an active SN1 branch, suggesting that in this yeast, KlSte11p may play a convergence point between the SHO1 and SN1 branches during the hyperosmotic stress response. Therefore, we decided to address the role of this MAPKKK protein in the K. lactis HOG pathway. For this purpose, we analyzed if in K. lactis KlSte11p can rescue a mutant lacking both branches of the HOG pathway from its sensitivity to hyperosmotic stress, as long as it is localized, somehow, in the plasma membrane. To this end, we fused the transmembrane domains of KlSho1p to the full KlSte11 protein, according to the scheme shown in Fig. 5A, and tagged it with a His6 epitope, which allowed us to confirm normal synthesis of the hybrid protein with anti-His antibody (not shown). We expressed the hybrid protein in the double Δklsho1 Δklssk2 mutant since it lacks the MAPKKK of the SLN branch and the protein that putatively recruits KlSte11p to the membrane.

We found that while the transmembrane KlSho1p segments [Sho1(TM)] alone did not restore the growth of the Δklste11 single mutant and the Δklsho1 Δklssk2 double mutant in high-salt medium (Fig. 5B), the Klsho1/Ste11 hybrid protein not only rescued the sensitivity shown by the Δklste11 mutant but also nicely rescued the Δklsho1 Δklssk2 double mutant. This indicates that KlSte11p can bypass the lack of KlSho1p.

Previously, it has been shown that KlSte11p plays an essential role in the mating pathway in K. lactis (25). Δklste11 mutants do not respond to sexual pheromone, and therefore, they show sterility. We tested if the fused Sho1/Ste11 protein may revert the sterile phenotype of the Δklste11 mutant. While the wild-type KlSte11 protein fully complemented the lack of endogenous KlSte11p, Klsho1/Klste11p failed to induce mating (Fig. 5C). This indicates that the fused protein is not available to participate in the pheromone response pathway.
FIG 5 KlSte11p attached to the plasma membrane rescues a ΔKlsho1 ΔKlssk2 mutant from its sensitivity to hyperosmotic stress. (A) Schematic representation of the fusion protein KlSho1/KlSte11. The four transmembrane segments of KlSho1p were fused to the N terminus of KlSte11p. (B) Expression of the KlSho1p/KlSte11 hybrid protein (within brackets) in ΔKlste11 or ΔKlsho1 ΔKlssk2 mutant strains. Cells carrying YeplKDgal alone or YeplKDgal with the indicated constructs were grown in Sgal medium until the OD600 was 0.4. Cells were collected, washed, suspended in fresh Sgal medium, and spotted as 10-fold dilutions onto YPD medium containing galactose instead of glucose or KCl. The plates were incubated at 30°C for 48 h and photographed. (C) Matting assay with a ΔKlste11 mutant expressing wild-type KlSte11p or the hybrid protein KlSho1p/Ste11 (within brackets). Patches of the indicated cells were grown on an Sgal plate for 24 h. The test strain was grown as a lawn on a YPD plate for 24 h. Both strains were replica plated onto YPD and incubated for 8 h at 30°C, allowing the cells to mate. Diploids were selected on SD me-}

 mutant produces sensitivity to high osmolarity in the same way that it has been observed in S. cerevisiae (4). Strikingly, we found that a lack of KLSte11p compromised growth on high-osmolarity medium, even with an intact SLN1 branch, indicating that Klste11p plays an essential role in the pathway responsible for the response to high stress. Accordingly, a mutant strain lacking KLSte50p was as sensitive to hyperosmotic stress as the ΔKLste11 mutant, suggesting that KLSte11p and KLSte50p may work in the hyperosmotic pathway as a complex in the same way that they do in the pheromone response system (25). The sensitivity to high stress of the ΔKLste11 mutant was as strong as that of mutants lacking the MAPKK KLPbs2p or the MAPK KIHog1p. Nevertheless, the presence of KLste11p by itself is not sufficient to induce osmoprotection, since a mutant devoid of the MAPKK KLssk2p and the adaptor protein KLSho1 is very sensitive to hyperosmotic stress. Since in S. cerevisiae, Sho1p has been proposed to recruit Ste11p to the membrane (8, 9), our results suggest that in K. lactis, the localization of KLSte11p is also crucial for its activity. Interestingly, when we targeted KLSte11p to the plasma membrane by means of adding the KlSho1p transmembrane segments to its N terminus, we were, in fact, able to generate hyperosmotic stress protection in a cell devoid of both KlSho1p and KLssk2p, indicating that under these conditions KLSte11p can bypass the requirement of the putative KlSho1p scaffolding activity. The Sho1p/Ste11p protein was not able to restore mating in a cell devoid of its endogenous KLSte11 protein, indicating that the chimeric protein was not available to participate in the pheromone response complexes. This would indicate that, besides its role in mating, Klste11p might be a common and essential component of the two HOG branches in K. lactis. It would be interesting to investigate the structural requirements for generation of an active complex, particularly those of KlSho1p, in order to recruit both KLSte11p and KLPbs2p. In light of our results, it appears that KLSte11p may play a core role allowing KLPbs2p to attach to membrane complexes, since the fused Sho1p/Ste11 protein, which lacks the KlSho1p docking domain for KLPbs2p, is able to generate osmoprotection.

In this work, we also found that exposure of K. lactis cells to high osmotic stress induced KIHog1p phosphorylation and its nuclear translocation. This response renders the cells osmoreistant and allows them to grow in hyperosmotic medium. To induce sensitivity to hyperosmotic stress in K. lactis, it was necessary to eliminate either the two HOG branches (SHO1 and SLN1) or the KLSte11p kinase. Elimination of the SHO1 branch at the level of the adaptor KLSho1p or the MAPKK KLSte11p and the SLN1 branch at the level of the MAPKK KLssk2p produced sensitivity to hyperosmotic stress. Surprisingly, in these mutants we observed KIHog1p phosphorylation. Even more, we also demonstrated that in the ΔKLste11 ΔKLssk2 double mutant, the KIHog1p activated under these conditions rapidly went into the nucleus but could not induce transcription of the glycerol-proton symporter gene KISTL1 or a protective response to the hyperosmotic shock, as would be expected. Additionally, our results showed that when the ΔKLste11 single mutant was exposed to hyperosmotic stress, KIHog1p was also phosphorylated, but the mutant was osmosensitive, as mentioned before. Interestingly, other single mutants, like the ΔKLsho1, ΔKLste20, and ΔKLssk2 mutants, that inactivate only one of the branches of the HOG pathway (SHO1 or SLN1) are able to induce KIHog1p phosphorylation and osmoreistance in cells exposed to osmotic stress. These results suggest that phosphorylation of KIHog1p in wild-type cells is somehow different from that of the ΔKLste11 single mutant and the ΔKLste11 ΔKLssk2 double mutant. A similar phenomenon has been observed in S. cerevisiae; in mutants devoid of any of the MAPKK protein (30) and in a double mutant lacking ScSte11p and the phosphorelay response regulator ScSek1p (31), ScHog1p gets phosphorylated and fails to induce osmoprotection against severe hyperosmotic stress (in medium with greater than 1.0 M NaCl or 0.8 M sorbitol). Maayan et al. (33) proposed that in S. cerevisiae, Hog1p might be autophosphorylated in mutants lacking the canonical SHO1 and SLN1 inputs, when they are exposed to highly severe osmotic
stress. Under our conditions, we did not observe the autophosphorylation of Hog1p in K. lactis and demonstrated that KlHog1p phosphorylation requires not only the presence of KlPbs2 protein but also its kinase activity.

Taken together, these observations suggest that in K. lactis the HOG response to hyperosmotic conditions can be activated in different ways, and in some of them, KlHog1p phosphorylation might be unrelated to the hyperosmotic response in this yeast species. Our results indicate that under certain circumstances, the phosphorylation of KlHog1p is done by an input different from the known Hog1 branches and that phosphorylation by this alternative pathway is unproductive for the osmotic response, since it does not induce protection, even though KlHog1p is translocated into the nucleus. It will be interesting to search for this putative alternative KlHog1p activator, one of which could be KlSte7p, since it was demonstrated that it participates in KlHog1p phosphorylation in cells lacking both the SHO1 and SLN1 branches, since the null mutant is sensitive to hyperosmotic stress but shows constitutive KlHog1p phosphorylation (26).

Finally, we do not discard the possibility that some other conditions, such as oxidative and heat stresses, may result in the activation of the K. lactis HOG pathway. In fact, enhanced KlHog1p phosphorylation has been shown in cells where KlPmr1p has been inactivated (34). Klpmr1 mutant cells exhibit a variety of defects, including altered calcium homeostasis and oxidative stress.

The findings obtained in this study contribute to an increase in the knowledge of the HOG pathways in different yeast species and confirm that Hog1p phosphorylation alone is not sufficient to induce a cellular response to hyperosmotic stress.

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