Protein Kinases Involved in Mating and Osmotic Stress in the Yeast Kluyveromyces lactis

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Received 2 October 2007/Accepted 29 October 2007

Systematic disruption of genes encoding kinases and mitogen-activated protein kinases (MAPKs) was performed in Kluyveromyces lactis haploid cells. The mutated strains were assayed by their capacity to mate and to respond to hyperosmotic stress. The K. lactis Ste11p (KlSte11p) MAPK kinase kinase (MAPKKK) was found to act in both mating and osmoresponse pathways while the scaffold KlSte5p and the MAPK KlFus3p appeared to be specific for mating. The p21-activated kinase KlSte20p and the kinase KlSte50p participated in both pathways. Protein association experiments showed interaction of KlSte50p and KlSte20p with Gα and Gβ, respectively, the G protein subunits involved in the mating pathway. Both KlSte50p and KlSte20p also showed interaction with KlSte11p. Disruption mutants of the K. lactis PBS2 (KlPBS2) and KLYOG1 genes of the canonical osmotic response pathway resulted in mutations sensitive to high salt and high sorbitol but dispensable for mating. Mutations that eliminate the MAPKK KlSte7p activity had a strong effect on mating and also showed sensitivity to osmotic stress. Finally, we found evidence of physical interaction between KlSte7p and KlHog1p, in addition to diminished Hog1p phosphorylation after a hyperosmotic shock in cells lacking KlSte7p. This study reveals novel roles for components of transduction systems in yeast.

Cellular signaling transduction networks continuously sense extracellular cues and transduce signals from the cell surface to the interior of the cell. The cell responds to these signals through changes in gene expression and protein activity to yield specific phenotypes.

G-protein signaling pathways in fungi are used to regulate development and virulence and to detect nutrients and other environmental signals. Some of these pathways contain mitogen-activated protein kinase (MAPK) cascades that are highly conserved in metazoans (12, 18). The yeast pheromone signaling, for example, occurs through the action of a G-protein-coupled receptor (GPCR) and the associated G protein in order to activate a MAPK cascade that conducts the signal to the nucleus. Besides G-protein-activated signal transduction pathways, there are at least three more pathways involving MAPK components or their presumed upstream regulators that modulate responses to several stimuli (10).

The best-characterized transduction system in eukaryotic organisms is the Saccharomyces cerevisiae pheromone response pathway. This pathway is initiated by the binding of peptide pheromones to a GPCR (Ste4p/Ste18p) and the associated G protein in order to activate a MAPK cascade that conducts the signal to the nucleus. Besides G-protein-activated signal transduction pathways, there are at least three more pathways involving MAPK components or their presumed upstream regulators that modulate responses to several stimuli (10).

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While a large amount of information has accumulated on signal transduction cascades in S. cerevisiae and in many other fungi (18), a critical question concerns which features of the
transduction systems are generic and which are species specific.
For example, in the budding yeast Kluyveromyces lactis, the signal
transduction system that mediates the mating response is triggered by both the Ga
(K. lactis Gap1p [KlGap1p]) (31) and Gβ (KlSte4p) (14) subunits of the heterotrimeric
G protein. There are two documented differences between the G protein pheromone response pathways of S. cerevisiae and K.
lactis: first, while disruption of GPA1, the gene encoding the G protein α-subunit in S. cerevisiae, leads to permanent growth arrest and therefore to lethality in haploid cells (7), inactivation of Ga (KlGap1p) in K. lactis does not affect cell viability but produces sterility (31); second, overexpression of the Gβ (Ste4p) subunit induces growth arrest and mating in S. cerevisiae (34) but has no effect in K. lactis (14). Although it has been shown that both Ga and Gβ trigger the mating response by activating the transcription factor KISte12p (14, 19), nothing is known about the elements that connect the G protein with the transcription factor in K. lactis. We have therefore investigated the phenotypic characteristics of disruption mutants in components of the pheromone response pathway, and in order to elucidate the overlapping of protein function with other transduction systems, we assayed the disruption mutants for their capacity to respond also to osmotic stress.

The budding yeast K. lactis provides an attractive model system for studying cellular differentiation processes in response to environmental cues. It is a unicellular and essentially aerobic and heterothallic organism with a conventionally organized cell cycle. Its entire genome sequence is now available (http://cbi.labri.fr/Genolevures), and it is easily subjected to genetic analysis.

**MATERIALS AND METHODS**

**Strains, media, and genetic techniques.** The yeast strains used in this work were K. lactis 155 (MATa ade2 his3 ura3) and 128b (MATa his4 arg4 ura3), S. cerevisiae strain EGY48 (MATa his3 pl1 ura3-52 leu2-lac3-lacZOP6) was used for two-hybrid assays (23). Escherichia coli strains DH5α and GM3 (for preparation of unmethylated DNA) were used to propagate plasmids. YPD medium consisted of 1% yeast extract, 2% Bacto-peptone, and 2% glucose. Synthetic dextrose (SD) minimal medium consisted of 0.67% yeast nitrogen base without amino acids (Difco) and 2% glucose. SD medium was supplemented with the required amino acids and nitrogen bases (50 μg/ml). S. cerevisiae strains containing the Gα subunit of the G protein pheromone response pathways of K. lactis triggered the mating response by overexpression of the Gβ (Ste4p) subunit, which induces growth arrest and mating in S. cerevisiae (34), but has no effect in K. lactis (14). Although it has been shown that both Gα and Gβ trigger the mating response by activating the transcription factor KlSte12p (14, 19), nothing is known about the elements that connect the G protein with the transcription factor in K. lactis. We have therefore investigated the phenotypic characteristics of disruption mutants in components of the pheromone response pathway, and in order to elucidate the overlapping of protein function with other transduction systems, we assayed the disruption mutants for their capacity to respond also to osmotic stress.

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**Gene identification and cloning.** A BLAST search of the K. lactis genome database, Genolevures project II (http://cbi.labri.fr/Genolevures/ell/KLLA), allowed the identification of putative open reading frames (ORFs) encoding the orthologues of S. cerevisiae protein kinases. Pairs a and b of the oligoodeoxynucleotides listed in Table 1 were used for PCR amplification. Chromosomal DNA from K. lactis strain 155 was used as a template. Standard PCR were carried out to amplify the desired gene fragments. The PCR products were cloned into the plasmid vector pPromega (Promega) and sequenced in full. Plasmid pPHO (containing the PHO5 promoter from S. cerevisiae and a K. lactis replication origin) was obtained from Hiroshi Fukuhara. A six-histidine tag was fused in frame to the C terminus of the K. lactis STE50 (KlSTE50) protein.

**Gene disruptions.** Gene disruptions were achieved by homologous recombination using the integrative vector YIp352 (11). Disruption mutants were selected by their capacity to grow in Ura-deficient medium. All gene fragments were obtained from the pGEMTeasy clones. For K. lactis STE50 (KlSTE50), a

**TABLE 1. Primers used for PCR amplification**

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence*</th>
</tr>
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<tbody>
<tr>
<td>Ste50a</td>
<td>(+88) CTGAGATAAGCCGAGACACAGAG (+114)</td>
</tr>
<tr>
<td>Ste50b</td>
<td>(+741) TTGATCTCAAGGTCATCGTAC (+715)</td>
</tr>
<tr>
<td>Ste50c</td>
<td>(+19) ATAGAAACAAAACACggATGACGGGTATC (+11)</td>
</tr>
<tr>
<td>Ste50d</td>
<td>(+929) AAGTTATTTTTCgtGAGTAGAGGCG (+899)</td>
</tr>
<tr>
<td>Ste20a</td>
<td>(+14) TTTGTCGCCagATGACGGCTACTGAT (+16)</td>
</tr>
<tr>
<td>Ste20b</td>
<td>(+3002) TAAATCTGACGATGAAACAGAAAT (+2972)</td>
</tr>
<tr>
<td>Ste5a</td>
<td>(+1) ATGTCGAGGTAATA (+17)</td>
</tr>
<tr>
<td>Ste5b</td>
<td>(+2300) GATTAGAGGAGAAGG (+2284)</td>
</tr>
<tr>
<td>Ste11a</td>
<td>(+369) GCCAGAAGAAGGTAAGTGAGG (+385)</td>
</tr>
<tr>
<td>Ste11b</td>
<td>(+1210) TGGACTCGAGAACG (+1192)</td>
</tr>
<tr>
<td>Ste11c</td>
<td>(+19) TTTTAAAGATCGAGACGGGTGAC (+12)</td>
</tr>
<tr>
<td>Ste11d</td>
<td>(+2222) AGTACAAATACCTGAGGACTGAAAAGG (+2194)</td>
</tr>
<tr>
<td>Ste7a</td>
<td>(+323) GGAAGGTCCTCGGTC (+247)</td>
</tr>
<tr>
<td>Ste7b</td>
<td>(+1803) CCTTTAAAGAGGTATCTTCCTCCT (+1275)</td>
</tr>
<tr>
<td>Ste7c</td>
<td>(+11) ATGATGACTAAAGTACGAGTATACG (+11)</td>
</tr>
<tr>
<td>Ste7d</td>
<td>(+1543) CTTATATAGTGCCATATATTTT (+1514)</td>
</tr>
<tr>
<td>Ste7e</td>
<td>(+1513) ccgcCTGACGTTGTTGTTGAT (+1501)</td>
</tr>
<tr>
<td>Fus3a</td>
<td>(+498) ACAGATGCGGCTTCGGATGGAG (+523)</td>
</tr>
<tr>
<td>Fus3b</td>
<td>(+1171) TGATATAAAGGCTGTGTTTATTAAG (+1144)</td>
</tr>
<tr>
<td>Pbs2a</td>
<td>(+21) GCCGATTAAGTGCACATTGAGT (+6)</td>
</tr>
<tr>
<td>Pbs2b</td>
<td>(+2293) GGCGAGGGAAGGTGCTTTACATCCGG (+2264)</td>
</tr>
<tr>
<td>Hog1a</td>
<td>(+490) CCGTCAGGATCCAGATCTCCTATTGAT (+517)</td>
</tr>
<tr>
<td>Hog1b</td>
<td>(+1366) CTTTCGTCGCGATATTTGACATC (+1393)</td>
</tr>
<tr>
<td>Hog1c</td>
<td>(+18) TTTTCAATTGGAAGATTTCTGCGAAGAT (+12)</td>
</tr>
<tr>
<td>Hog1d</td>
<td>(+1397) TACTATCTTCGCTCCATTCATTCAT (+1343)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses correspond to coordinates considering A in the translation start codon as the +1 position. Nucleotide changes introduced to generate restriction sites or tag sequences are indicated by lowercase letters.

SmaI-HindIII 634-bp fragment was introduced into the integrative vector YIp352 previously digested with EcoRI (Klenow filled) and HindIII. The resulting plasmid was digested with EcoRI and SacI (natural restriction sites of KISTE50 ORF located at positions 296 and 438, respectively). This yields a linear YIp352 plasmid flanked by 298 bp and 196 bp of KISTE50 recombinant ends. For KISTE72, a 1,046-bp fragment obtained by EcoRI and SacI digestion was cloned into YIp352 digested with the same enzyme. The resulting integrative plasmid was opened with BglII, which cuts the KISTE20 ORF at position 268. The resulting construct contains recombinant ends of 267 bp and 779 bp. KISTE50 was obtained as a 1,266-bp EcoRI fragment and ligated into YIp352 opened with the same enzyme. The resulting construct was digested with Hpal and SpeI, yielding an integrating molecule with 573 bp and 140 bp of recombinant ends. A KISTE11 850-bp fragment obtained with HindIII-Asp718 was introduced into YIp352 digested with the same enzymes. This plasmid was opened with SpeI, yielding an integrating fragment containing 190 bp and 275 bp of recombinant ends. For KISTE77, a 1,071-bp fragment was obtained by HindIII-BamHI digestion and ligated into YIp352 digested with the same enzymes. The resulting construct was digested with XbaI and BglII, leaving recombinant ends of 333 bp and 338 bp. For KIP352, a 670-bp EcoRI fragment was subcloned into YIp352 vector prepared with the same enzyme. A linear molecule was generated by digesting the resulting plasmid with SmaI and PstI. This yields an integrating molecule containing 227 bp and 277 bp of recombinant ends. A 967-bp KpnI-PstI fragment of KIPBS2 was subcloned into YIp352 opened with the same enzymes. The YIp352-
KIPBS2 construct was digested with XbaI and BstEII, yielding a linear molecule containing 431 bp and 313 bp of recombinant ends. For KIHOG1, a BamHI-HindIII 876-bp fragment was subcloned into YIplac352 digested with the same enzymes. BglII digestion of Ylp532-KIHOG1 yielded a linear molecule flanked by recombinant ends of 331 bp and 350 bp. When required, YPD medium containing 1 mg/ml of 5-fluorouracil (5-FU) was used for negative selection of the URA3 cassette.

**Protein interactions.** Assays of physical interaction were done with a LexA-B42 two-hybrid system as described previously (14). The KIGP1A and KISTE4 genes were subcloned into pEG202 as previously reported (14, 31). KIGP1A was also subcloned into pG4-5 as reported previously (14). Genes of interest (with the exception of KISTE20 and KIPBS2) were amplified by PCR employing specific c and d primers (Table 1) and ligated in frame into two-hybrid plasmids. The full KISTE20 gene was ligated into pG4-5 as an XhoI fragment. KISTE11 was subcloned into pEG202 as a 2,215-bp Xhol fragment. The KISTE7 gene was obtained as a 1,536-bp Xhol fragment and ligated into pG4-5. The gene encoding KIPBS2p was subcloned into pEG202 as a 2,285-bp Asp718-BamHI fragment. KIHOG1 was subcloned in both pEG202 and pG4-5 as a 1,364-bp EcoRI-Xhol fragment. The KISTE20 gene was obtained as a 2,987-bp fragment from the S. cerevisiae endochitinase gene (CTS1) cloned into either plasmid pEG202 or pG4-5 (14) was used as a negative interaction control for each interacting couple. All recombinant genes were sequenced in full. S. cerevisiae strain EGY48 was transferred with two-hybrid plasmids and grown overnight in SD medium at 30°C. Induction of genes subcloned into pG4-5 was done by shifting cells to SGal medium 4 h prior to harvesting. Protein interactions were determined by the ability of hybrid proteins to induce blue colonies by expression of the LACZ reporter gene located in the pSH1-54 plasmid and by reversion of the leu2 deficiency.

**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 a YPD plate and incubated for 8 h at 30°C, allowing cells to mate. Diploids were selected on SD medium by replica plating.

**Osmotic stress assays.** Strains to be tested were grown overnight on SD medium. Cells were washed, suspended in YPD medium at an optical density at 600 nm of 0.1, and grown to mid-exponential phase. Serial dilutions of the samples were spotted on YPD medium containing 0.5 M KCl or 1 M d-sorbitol and incubated at 30°C. Plates were photographed 48 h later.

**Phosphorylation assays.** To estimate the cellular content of both total and phosphorylated KIHog1p in wild-type, ΔKIPBS2, and ΔKISTE7 strains, exponentially growing cells in 5 ml of YPD medium were pelleted and suspended in YPD medium or YPD medium plus 0.5 M KCl for different times. Cells were concentrated by centrifugation, and the supernatant was removed by aspiration. Cells were resuspended in loading buffer (5% sodium dodecyl sulfate [SDS], 0.1 M Tris-HCl [pH 7.5], 5% glycerol, 0.07 M 2-mercaptoethanol, 0.02 mM bromophenol blue) and boiled for 5 min. Samples were centrifuged, and the supernatants were transferred to a clean tube. An aliquot of the samples was subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel and transferred to polyvinylidene difluoride membrane (Millipore Corporation).

**Immunoblotting.** Blots were incubated with blocking buffer (1% phosphate-buffered saline, 1% albumin, 0.05% Tween 20) for 1 h at 37°C. Blots were incubated in the same buffer containing rabbit polyclonal anti-Hog1p (Santa Cruz Biotechnology) or rabbit monoclonal anti-phanos-p80 antibody (Cell Signaling Technology) for 1 h at room temperature. Filter-bound antibodies were detected with horseradish peroxidase-conjugated secondary goat-anti-rabbit immunoglobulin G antibody (Zymed) and visualized with chemiluminescent horse-radish peroxidase antibodies (Millipore Corporation).

**Immunoprecipitation assays.** Cells bearing pPHO or pPHO-kiStel8 (His5) were grown at 30°C to mid-log phase in 50 ml of selective medium. At mid-log phase, KI-P04 was added at a final concentration of 10 mM, and cells were incubated for another 2 h at 30°C. Half of the culture was treated with 0.5 M KCl for 10 min. Cells were collected by centrifugation and resuspended in 300 μl of TEA buffer containing 10 mM Tris (pH 7.4), 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, 20 mM NaN3, and 1× complete protease inhibitor cocktail (Roche). Sterile acid-washed glass beads were added, and cell disruption was performed by vortexing for 5 min at 4°C. Lysates were cleared into a microcentrifuge (3 min at 20,000 rpm), and the supernatant was adjusted to an 800-μl final volume with TEA buffer. Ten microliters of anti-His6-peroxidase antibody (Roche) was added and incubated at 4°C overnight with gentle shaking. Sixty microliters of protein G-agarose beads (Upstate) was added, and incubation continued for 2 h. The beads were washed three times with 1.0 ml of cold TNTE buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA). Thirty microliters of loading buffer was added, followed by 5 min of boiling. Samples were chilled on ice and centrifuged briefly. Twenty microliters of each sample was used for immunoblotting. Blots were probed with anti-Hog1p as described above. Reciprocal immunoprecipitation was done in the same way except that 5 μl of anti-Hog1p (Santa Cruz Biotechnology) antibodies was used for precipitation, and blots were probed with anti-His6-peroxidase antibodies.

**RESULTS**

In previous work, we have demonstrated that both the Gas (KIGP1A) and Gβ (KISTE4) subunits of the heterotrimeric G protein trigger the mating pheromone response pathway in K. lactis (6, 14, 31). Additionally, we have found that this activation requires the transcription factor KISIe12p (14, 19). In order to elucidate further the pathway that promotes mating in K. lactis, we constructed disruption mutants of genes encoding putative intermediates between the G protein and the KISIe12p transcription factor. We therefore inactivated the genes encoding components of the MAPK cascade, and we also inactivated the genes for KISIe50p and KISIe20p protein kinases, two putative inputs of the MAPK module. To investigate the overlapping of protein function with other transduction systems, we assayed the disruption mutants for their capacity to respond to osmotic stress, and for this purpose we added to this screening mutants of genes encoding KIPBS2 and KI-Hog1p, which have been shown to be key elements in the osmotic adaptation response in other yeast species (10, 15). Table 2 shows relevant features of the studied genes and their products as well as the degrees of homology with their S. cerevisiae counterparts.

Because each particular protein may modulate responses in different degrees and its inactivation may have several effects, detailed studies on the structure and the role that each protein may have in different cell functions will be described elsewhere.

All disruption mutants were constructed by integrating a URA3 cassette in haploid cells of both mating types; therefore, transformants were selected by their capacity to grow in Ura-deficient medium, and the disrupted alleles were confirmed by

Table 2. Proven components of K. lactis mating and osmoregulatory pathways

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Mutant phenotype</th>
<th>S. cerevisiae homologue</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>KISTE2</td>
<td>Sterile</td>
<td>ND</td>
<td>48</td>
</tr>
<tr>
<td>KISTE3</td>
<td>Sterile</td>
<td>ND</td>
<td>51</td>
</tr>
<tr>
<td>KIGP1A</td>
<td>Sterile</td>
<td>ND</td>
<td>62</td>
</tr>
<tr>
<td>KISTE4</td>
<td>Sterile</td>
<td>ND</td>
<td>52</td>
</tr>
<tr>
<td>KISTE18</td>
<td>Fertile</td>
<td>ND</td>
<td>53</td>
</tr>
<tr>
<td>KISTE20</td>
<td>Sterile</td>
<td>Sensitive</td>
<td>50</td>
</tr>
<tr>
<td>KISTE50</td>
<td>Sterile</td>
<td>Sensitive</td>
<td>41</td>
</tr>
<tr>
<td>KISTE5</td>
<td>Sterile</td>
<td>Resistant</td>
<td>27</td>
</tr>
<tr>
<td>KISTE11</td>
<td>Sterile</td>
<td>Sensitive</td>
<td>58</td>
</tr>
<tr>
<td>KISTE7</td>
<td>Sterile</td>
<td>Sensitive</td>
<td>52</td>
</tr>
<tr>
<td>KIPUS3</td>
<td>Sterile</td>
<td>Resistant</td>
<td>69</td>
</tr>
<tr>
<td>KISTE12</td>
<td>Sterile</td>
<td>ND</td>
<td>35</td>
</tr>
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<td>KIPBS2</td>
<td>Fertile</td>
<td>Sensitive</td>
<td>57</td>
</tr>
<tr>
<td>KIHOG1</td>
<td>Fertile</td>
<td>Sensitive</td>
<td>80</td>
</tr>
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</table>

a ND, not determined.

b Identity values were determined by BLAST using the BLOSUM62 matrix.
Southern blot. In all cases, plasmidic gene copies reversed the pheno-
typic defects of 5-FOA-resistant strains to almost wild-
type levels (data not shown). Since our results suggest that
gene disruptions seem to have the same phenotypic effects in
MATα and MATα cells, results described in this work refer
solely to MATα strains.

All mutant strains were viable, and they exhibited normal
growth; however, when assayed in sexual crosses with a tester
strain, they showed (with the exception of Klhog1 and Klpbs2
mutants) mating defects (Fig. 1). The hierarchy of defects in
mating was determined as follows: Klste5 = Klste20 = Klste11 =
Klste7 > Klste50 > Kl fus3 (Fig. 1). The strongest effect on
mating was observed when the scaffold protein KLSte5p and
the KLSte20p, KLSte11p, and KLSte7p kinases were inactivated,
indicating that all these proteins play essential roles in haploid
mating. KLSte5p shows a moderate degree of identity with its
counterpart in S. cerevisiae (27% identity) (Table 2). S. cerevisiae
Ste5p (ScSte5p) has no catalytic activity but acts as a
scaffold, organizing and forming a complex with the MAPK
module composed by ScSte11p, ScSte7p, and ScFus3p (4, 35).
The KLSte20p is a member of the PAK family (17), which is
50% identical to ScSte20p (Table 2) and shows high degree of
conservation in the CRIB domain, needed for Cdc42p binding
(a small Rho-like G protein) (1), and in the G protein
(focused of this analysis because we have observed that they are
implicated in the pheromone response pathway, possibly acting
as effectors of the G protein (data not shown). The two-hybrid
assay detected that KLSte50p makes a moderate interaction
with KGpa1p, the Go subunit of the heterotrimeric G protein,
while the KLSte20p kinase interacts strongly with the Gβ sub-
unit KLSte4p (Fig. 2). These assays also revealed that both

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**FIG. 1.** Mating of MATα disruption mutants. Mating was done by
replica plating. Plates of strains to be tested were streaked on selec-
tive medium and replicated onto YPD plates containing a lawn of the
MATα wild-type tester strain, followed by incubation overnight at 30°C.
Diploid selection was done by replica plating onto SD medium. Plates
were photographed 48 h later. WT, wild type.

**FIG. 2.** Protein interactions determined by the two-hybrid system.
The binding domain corresponds to LexA-fused proteins cloned into
pEG202. The activation domain corresponds to B42-fused proteins
cloned into pJG4-5. Two-hybrid plasmids were introduced into strain
EGY48. β-Galactosidase activity was determined by the relative
intensity of blue staining in cells growing on SGal plates containing 1 mg/ml
X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), pH 7.0.
Growth in SD medium without leucine (leu−) was determined by
relative size of the colony after a 24-h incubation at 30°C. The S.
cerevisiae endochitinase gene (CTS1) cloned either into pEG202 or
pJG4-5 was used as negative interaction control for each interacting
couple.
the KlSte50p and the KlSte20p kinases associate with the MAPKKK KlSte11p. The KlSte50p-KlSte11p interaction is stronger than that of KlSte20p with KlSte11p (Fig. 2), most probably due to the presence of the strongly interacting SAM domains in KlSte50p and KlSte11p (30). These findings suggest that KlSte50p and KlSte20p may serve as links between the G protein and the MAPK module in the transmission of the pheromone stimulus in K. lactis.

Several elements of the yeast pheromone response pathway play a role in other signal transduction cascades (10, 13, 20). Concerning the high-osmolarity response pathway in S. cerevisiae, at least three elements of the pheromone pathway are needed to regulate the osmoadaptation program: Ste50p, Ste20p, and Ste11p, which function as upstream elements of the scaffold and kinase protein Pbs2p and the MAPK Hog1p in one of the branches dedicated to adaptation of yeast to high osmolarity (13). All these proteins are components of the so-called HOG pathway. We assayed the K. lactis disrupted strains under conditions that produced osmotic (high K⁺ and high sorbitol) stress (Fig. 3). In this set of experiments we found increased sensitivity to sorbitol and KCl of mutant strains ΔKlpbs2 and ΔKlhog1 compared to the wild-type strain, which is in agreement with their expected roles in osmoadaptation. The strong sensitivity of mutant ΔKiSte7 to high osmolarity was also evident, indicating some parallelism of KiSte11p function with Ste11p from S. cerevisiae (26). Surprisingly, in this set of experiments we observed that the ΔKiSte7 mutant showed sensitivity to stress conditions, as indicated by its marked growth delay in 0.5 M KCl and 1 M sorbitol compared to the growth of wild-type cells (Fig. 3). This finding is striking and suggests that the MAPKK KiSte7p is involved in a signal transduction cascade related to the osmotic response. Inactivation of the KlSte50p and KlSte20p kinases produced severe sensitivity to high osmotic stress, confirming that these kinases play overlapping roles in the propagation of different signals (Fig. 3). As expected, the ΔKiSte5 and ΔKiJus3 mutants were insensitive to hyperosmotic stress (Fig. 3).
reduced but did not eliminate mating. Our experiments produced complete sterility while disruption of KLSte50p reduced mating but does not eliminate it (31). The physical association between Gα and KLSte50p opens a new window for the study of new functional relationships of G protein signaling components.

Participation of Ste50p, Ste20p, and Ste11p in the Sho1p branch of the HOG pathway has been extensively documented in S. cerevisiae (12, 15). In a brief view of the pathway, the osmosensor Sho1p recruits Pbs2p to the membrane during signaling. Both Sho1p and Pbs2p can bind Ste11p. Ste11p is activated by phosphorylation, which is mediated by the Ste20p and Cla4p kinases and requires Ste50p (30). Ste11p activates Pbs2p, which in turn activates Hog1p (15). The high-osmolarity response pathway in S. cerevisiae is also regulated by the Sln1p branch, which consists of the Sln1p-Ypd1p-Ssk1p phosphorelay system. Sln1p is an osmosensor histidine kinase, Ypd1p is a phosphotransfer protein, and Ssk1p is a response regulator (25). Hyperosmotic shock deactivates Sln1p, leading to activation of Ssk2p and Ssk22p (two redundant kinases) via the Ssk1p response regulator. Ssk2p and Ssk22p activate the MAPKKK Pbs2p, which in turn activates, by phosphorylation, the MAPK Hog1p (22). The two branches for Hog1p activation are not redundant, since the Sln1p-Ssk1p branch has a more prominent role than the Sho1p-Ste11p branch in inter-branch osmosensitivity (0.1 M KCl), but both branches function at high osmolarity (24). At present, it is not known if these two branches are active in K. lactis; however, the phenotype of strong sensitivity to high sorbitol (1.0 M) and high salt (0.5 M KCl) displayed by mutants ΔKLSte50, ΔKLSte20, and ΔKLSte11, strongly suggests that the Sho1p pathway has remarkable participation in osmoadaptation to high-stress conditions. Moreover, disruption of KISTE11 induces severe growing defects under high osmotic stress in K. lactis, while in S. cerevisiae cells, inactivation of Ste11p induces sensitivity to hyperosmotic conditions only in Δsks1 or Δsks2 Δsks22 backgrounds (22, 24). In addition, it is worth noting that the Sln1p branch of the HOG pathway in K. lactis lacks the homologue Ssk22p (15) although we actually do not know the functional meaning for the absence of this MAPKK.

In addition to the interaction detected between components of the MAPK module and scaffold proteins, two-hybrid studies indicate that some of the protein kinases associate with each other independently of their association with the scaffold protein. In particular, both ScSte7p and ScSte11p interact with the MAPKs ScFus3p and ScKss1p, independently of Ste5p (4, 29). However, due to the sensitivity to conditions of high osmotic stress and to the lower KIIHog1p phosphorylation level displayed by the ΔKLSte7 strain, we believe that the interaction observed between KLSte7p and KIIHog1p is significant and might play an important role in osmoadaptation. In S. cerevisiae, the Ste7p kinase has been shown to participate not only in mating but also in filamentous and invasive growth (20); however, a mutant strain lacking Ste7p shows normal osmotolerance, which indicates that this kinase does not participate in the HOG response pathway in this yeast species. We were unable to find physical interaction between KLSte7p and KIPbs2p, and the interaction between KIPbs2p and KIIHog1p was rather weak. It is well known that failure to find an association by the two-hybrid system can occur for many reasons; therefore, association between these proteins should be tested by different techniques.
Sensitivity of the ΔKlste7 mutant to high osmotic stress and association of KlSte7p with KlHog1p in K. lactis are, at least, intriguing observations. Either KlPbs2p lacks a kinase activity and KlSte7p is able to replace it, or KlHog1p can be phosphorylated by redundant MAPKK in the HOG pathway. Our results discarded the first hypothesis since KlHog1p phosphorylation is still observed in ΔKlste7 mutant cells. Although we have no direct evidence that KlSte7p phosphorylates KlHog1p, we found that the amount of phosphorylated KlHog1p diminished in cells lacking the KlSte7p kinase. This may affect the growing rate of the Klste7 mutant under hyper-osmotic conditions. Additionally, our results showed that KlHog1p phosphorylation is completely dependent on KlPbs2p, suggesting that KlHog1p needs to be recruited by the scaffold activity of KlPbs2p in order to be phosphorylated by KlSte7p; i.e., KlSte7p fails to phosphorylate soluble KlHog1p.

We have provided evidence that in addition to KlSte50p, KlSte20p, and KlSte11p, KlSte7p also participates in both the pheromone response pathway and in the high-osmolarity response pathway in K. lactis. Despite the common use of protein kinases in both pathways, our observations also indicate that there is no cross talk between these two signaling systems; i.e., sexual pheromone does not induce a high-osmolarity response and conditions of high osmotic stress do not induce mating (data not shown).

The composition of the G protein signaling pathway for mating, the participation of the KlSte7p in the HOG pathway, and the strong sensitivity phenotype of the ΔKlste11 mutant to hyperosmotic stress indicate that signaling systems in K. lactis follow, at least in part, mechanisms different from those of S. cerevisiae. The perspective ahead is to understand further the organization and functional interactions of the elements belonging to the intracellular MAPK signaling pathways in K. lactis. The results will be of interest for the understanding of related problems in other organisms.

ACKNOWLEDGMENTS

We thank Guadalupe Codiz and Minerva Mora (Molecular Biology Unit) for technical assistance and the staff of the Computer Facility at Instituto de Fisiologia Celular, Universidad Nacional Autónoma de México. We also thank Marisela Bolaños for technical assistance.

This work was partially funded by grants 44178 from CONACyT, México, and IN211906 from PAPIIT, DGAPA, Universidad Nacional Autónoma de México.

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