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The Osmoregulatory Pathway Represses Mating Pathway Activity in *Saccharomyces cerevisiae*: Isolation of a *FUS3* Mutant That Is Insensitive to the Repression Mechanism

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Mitogen-activated protein (MAP) kinase cascades are conserved signal transduction pathways that are required for eukaryotic cells to respond to a variety of stimuli. Multiple MAP kinase pathways can function within a single cell type; therefore, mechanisms that insulate one MAP kinase pathway from adventitious activations by parallel pathways may exist. We have studied interactions between the mating pheromone response and the osmoregulatory (high-osmolarity glycerol response [HOG]) pathways in *Saccharomyces cerevisiae* which utilize the MAP kinases Fus3p and Hog1p, respectively. Inactivating mutations in HOG pathway kinases cause an increase in the phosphotyrosine content of Fus3p, greater expression of pheromone-responsive genes, and increased sensitivity to growth arrest by pheromone. Therefore, the HOG pathway represses mating pathway activity. In a *HOG1*$^+$ strain, Fus3p phosphotyrosine increases modestly and transiently following an increase in the extracellular osmolarity; however, it increases to a greater extent and for a sustained duration in a *hog1*-Δ strain. Thus, the HOG-mediated repression of mating pathway activity may insulate the mating pathway from activation by osmotic stress. A *FUS3* allele whose gene product is resistant to the HOG-mediated repression of its phosphotyrosine content has been isolated. This mutant encodes an amino acid substitution in the highly conserved DPXDEP motif in subdomain XI. Other investigators have shown that the corresponding amino acid is also mutated in a gain-of-function allele of the MAP kinase encoded by the *rolled* locus in *Drosophila melanogaster*. These data suggest that the DPXDEP motif plays a role in the negative regulation of MAP kinases.

A variety of extracellular stimuli activate protein kinase cascades in eukaryotic cells. Some of these cascades lead to activation of the serine/threonine-specific, mitogen-activated protein (MAP) kinases (for reviews, see references 3, 7, 14, 17, and 28). MAP kinases are activated by phosphorylation of a conserved tyrosine and a conserved threonine in their catalytic clefts. This is accomplished by a dual-specificity MAP kinase kinase, or MEK (MAP/ERK kinase). MEKs are phosphorylated on serine and threonine residues by an activated MEK kinase. This sequential activation of MEK kinase, MEK, and MAP kinase constitutes the MAP kinase module (40). These modules are positioned downstream of a variety of signalling molecules, including receptor tyrosine kinases, serpentine receptors, and homologs of bacterial two-component systems. MAP kinase substrates include transcriptional and cell cycle regulatory proteins which effect cellular responses appropriate to the input signal.

Several MAP kinase-mediated pathways have been identified in the budding yeast *Saccharomyces cerevisiae*. The most thoroughly studied of these is the mating pheromone response pathway (3, 28) (see below). At least three of the kinases in the mating pathway are also involved in starvation-induced pathways that trigger a switch to pseudohyphal- or invasive-type growth (34, 43). Thus, components of MAP kinase modules can be utilized by different pathways for different responses. The high-osmolarity glycerol response (HOG) pathway is activated by an increase in the extracellular osmolarity (10, 35, 36) (see below). The pathway that uses the MAP kinase Mpk1p (also called Slt2p [38]) provides a response to low extracellular osmolarity and ensures the proper construction of cell wall components (19, 30, 32). The MAP kinase Smk1p is required for the completion of spore development (31).

The mating cascade induces a specialized differentiation program leading to mating competence and, ultimately, formation of the zygote. Haploid budding yeast exists in one of two mating types, either **a** or α, and the mating cascade is initiated when the secreted pheromone from one cell type is bound by its serpentine receptor on the surface of the opposite cell type. The subsequent activation of a heterotrimeric G protein results in activation of the Ste20p kinase via a mechanism that requires the GTP-binding protein Cdc42p (50, 58). This is required for the activation of a MAP kinase module consisting of the MEK kinase Ste11p, the MEK Ste7p, and the partially redundant MAP kinases Fus3p and Kss1p. Activated Fus3p phosphorylates Far1p, which then binds to and inhibits the cyclin-Cdc28 kinase complexes and causes G_1 arrest (15, 24, 41). Activated Fus3p and Kss1p phosphorylate the transcription factor Ste12p, which binds to pheromone-responsive DNA elements in the promoters of mating-specific genes and induces their expression. The products of these mating-specific genes are then required for fusion of the yeast cell with its mating partner and formation of the **a**/α diploid (see references 3 and 28 and references therein for reviews of the mating cascade). The G_1 arrest and differentiated morphology that

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TABLE 1. Yeast strains used

Strain[a]	Genotype	Reference or source
YPH102a	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} leu2-Δ1 his3-Δ200*	11
MAY1	*MAT***a** *ura3-52 lys2-801^{amber} ade2-101^{ochre} leu2-Δ1 his3-Δ200 pbs2-Δ2::LEU2*	M. Gustin
EY957	*MAT***a** *sst1Δ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100*	24
EY966	*MAT***a** *sst1Δ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 fus3-6::LEU2 kss1::HIS3*	E. Elion
JPHY10	*MAT***a** *sst1Δ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 fus3-6::LEU2 kss1::HIS3 lys2::FUS1-lacZ*	This study
JPHY11	*MAT***a** *sst1Δ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 fus3-6::LEU2 kss1::HIS3 lys2::FUS1-lacZ hog1::TRP1*	This study
JPHY12	*MAT***a** *sst1Δ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 fus3-6::LEU2 kss1::HIS3 lys2::FUS1-lacZ far1::TRP1*	This study
JPHY13	*MAT***a** *sst1Δ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 fus3-6::LEU2 kss1::HIS3 lys2::FUS1-lacZ msg5-2*	This study
JPHY14	*MAT***a** *sst1Δ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 fus3-6::LEU2 kss1::HIS3 lys2::FUS1-lacZ hog1::TRP1 msg5-2*	This study
JPHY15	*MAT***a** *sst1Δ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 fus3-6::LEU2 kss1::HIS3 lys2::FUS1-lacZ ptp2::hisG*	This study
JPHY16	*MAT***a** *sst1Δ ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 fus3-6::LEU2 kss1::HIS3 lys2::FUS1-lacZ hog1::TRP1 ptp2::hisG*	This study
EY1118	*MAT***a** *sst1Δ ade2-1 his3-Δ200 leu2-3,112 trp1-1 ura3-1 can1-100 lys2::FUS1-HIS3*	E. Elion
CY196	*MAT***a** *sst1Δ ade2-1 his3-Δ200 leu2-3,112 trp1-1 ura3-1 can1-100 lys2::FUS1-HIS3 hog1::LEU2*	This study

[a] All strains are coisogenic derivatives of the W303 genetic background except for YPH102a and MAY1, which are coisogenic strains in the S288C background.

result from mating pathway activation make it essential that the basal state of this pathway is tightly controlled.

The osmoregulatory, or HOG, pathway is required for the physiological response of a yeast cell to an increase in the extracellular osmolarity. The HOG pathway module includes the redundant MEK kinases Ssk2p and Ssk22p, the MEK Pbs2p, and the MAP kinase Hog1p. Pbs2p appears to integrate two distinct upstream signals. One signal comes from a two-component system consisting of the transmembrane sensor histidine kinase Sln1p and the cytoplasmic response regulator Ssk1p (36). The other signal derives from the transmembrane osmosensor Sho1p, and the carboxy-terminal tail of Sho1p has been proposed to interact directly with Pbs2p to facilitate its activation (35). Activation of Hog1p leads to the up-regulation of several genes whose products allow the cell to respond to the osmotic stress, and one class of HOG-responsive genes encodes enzymes that are rate limiting in glycerol biosynthesis. Cytoplasmic glycerol concentrations increase in response to high extracellular osmolarity, and the osmotic gradient across the plasma membrane is restored. Thus, the HOG pathway activates a physiologically graded response to a constant stimulus (i.e., a deviation from osmotic equilibrium).

The existence of multiple MAP kinase cascades within a single cell type and the homology among the different kinases at each tier of these cascades suggest that the activation of one MAP kinase pathway might lead to inappropriate activation events in parallel MAP kinase pathways. Mechanisms that insulate a given MAP kinase pathway from these inappropriate activations may therefore exist. In this report, we show that the mating pheromone response pathway is negatively regulated by the HOG pathway. Using a genetic screen, we isolated a *FUS3* mutant which encodes a MAP kinase that bypasses the HOG-mediated repression of its phosphotyrosine content. We propose that repression mechanisms such as these may serve to insulate MAP kinase-mediated cascades from adventitious activations that could lead to inappropriate cellular responses.

MATERIALS AND METHODS

Yeast strains and culture conditions. The genotypes and sources of strains used in this study are shown in Table 1. Cultures were maintained on YPD (1% yeast extract, 2% Bacto Peptone, 2% glucose) or on SD (0.67% Difco yeast nitrogen base without amino acids and 2% glucose) supplemented with essential nutrients as specified (45). Cells were grown to 1×10^7 to 2×10^7/ml at 30°C in YPD and were osmotically stressed by adding 5 M NaCl or 4 M D-sorbitol to a final concentration of 0.5 or 0.7 M, respectively. For α-factor treatment, cells were grown to 1×10^7 to 2×10^7/ml at 30°C in YPD, and α-factor (Sigma Chemical Co.) was added to 1 μM from a 1 mM stock in methanol. Unless indicated otherwise, α-factor treatments lasted 60 min at 30°C. Growth arrest by mating pheromone was assessed by spotting dilutions of overnight cultures onto YPD plates containing 0, 3.5, 7, and 14 nM α-factor. When plasmid maintenance was required, culture dilutions were spotted onto selective plates containing 0, 5, 10, 15, and 25 nM α-factor. The *FUS1-lacZ* pheromone-inducible reporter was integrated at the *LYS2* locus by using plasmid pJB230 (12). *HOG1* was disrupted with *TRP1* as described previously (10). This disrupts *HOG1* at codon 64 in the open reading frame (ORF) and removes 402 bp in the coding region. For strain CY196, *HOG1* was disrupted by using *Sal*I- and *Bam*HI-digested pDHG14 which contains *LEU2* inserted at the *Dra*I and *Fsp*I sites of *HOG1* (H. Saito, Dana-Farber Cancer Institute). This disrupts *HOG1* at codon 26 in the ORF and removes 1,083 bp in the coding region. *FAR1* was disrupted with the *URA3* gene by using *Not*I-digested pFC13 (15), and the integrated *URA3* locus was subsequently disrupted with *TRP1* by using *Kpn*I- and *Sal*I-digested pJO119 (41). The Far1$^-$ phenotype was confirmed by testing for the multilobed morphological response to α-factor as described previously (15).

Plasmid constructions. The plasmids used in this study are described in Table 2. To construct the constitutive *FUS3* expression plasmid pJPH10, the 600-bp *Hin*dIII-*Bam*HI fragment from pG-3 that contains the constitutive glyceraldehyde-3-phosphate dehydrogenase gene (*GPD*) promoter (46) was first cloned into these sites in pRS316 to create pJPH11. The *FUS3* ORF was amplified by the PCR using primers that introduced a *Bam*HI site at position −1 relative to the initiator ATG and an *Xba*I site at position +1325 and then ligated into these sites in pJPH11 to generate pJPH10. To create pJPH13 and pJPH14, the 2.6-kb *Bsp*DI-*Bam*HI fragments from pJB30 and pJB32 containing wild-type *HOG1* and the *hog1^{K52R}* mutant (47), respectively, were first ligated into YCp50 (44) at those sites. The large fragments from these constructs generated by partial *Sal*I followed by complete *Xma*I digestions were then ligated to the *Sal*I-*Xma*I fragment from YDpK that contains the *LYS2* gene (4). pJPH15 was created by digesting pJPH13 with *Eco*RI (to remove all of the *HOG1* promoter in that construct, as well as the first 168 codons in the *HOG1* ORF) followed by intramolecular ligation. Plasmids pJPH12, -22, -23, and -24 were constructed by

TABLE 2. Plasmids used

Plasmid	Markers	Reference
pRS316	*CEN URA3*	49
pJPH10	*CEN URA3 GPD-FUS3*	This study
pJPH11	*CEN URA3 GPD*	This study
pJPH12	*CEN URA3 FUS3*	This study
pJPH13	*CEN LYS2 HOG1*	This study
pJPH14	*CEN LYS2 hog1^{K52R}*	This study
pJPH15	*CEN LYS2 hog1-Δ*	This study
pJPH16	*CEN URA3 GPD-FUS3^{Y182F}*	This study
pJPH17	*CEN URA3 GPD-FUS3^{D317G}*	This study
pJPH18	*CEN URA3 GPD-FUS3^{F329S}*	This study
pJPH19	*CEN URA3 GPD-FUS3^{D317G,F329S}*	This study
pJPH20	*CEN URA3 GPD-FUS3^{L63P}*	This study
pJPH21	*CEN URA3 GPD-FUS3^{D317N}*	This study
pJPH22	*CEN URA3 FUS3^{D317G}*	This study
pJPH23	*CEN URA3 FUS3^{F329S}*	This study
pJPH24	*CEN URA3 FUS3^{D317G,F329S}*	This study
pYEE81	*CEN URA3 FUS3*	23

digesting pJPH10, -17, -18, and -19 (see below) with *Kpn*I and *Bgl*II, eliminating the *GPD* promoter and *FUS3* sequence 5′ to the *Bgl*II site, and ligating in the *Kpn*I-*Bgl*II fragment of a *FUS3* PCR product generated with a primer that introduced a *Kpn*I site at position −450 relative to the initiator ATG. Codons 329, 63, and 317 in the *FUS3* ORF of pJPH10 were altered by oligonucleotide-directed mutagenesis (Clontech Laboratories Inc.) to create the point mutations in pJPH18, -20, and -21, respectively. Codon 182 in the *FUS3* ORF in pYEE114 was altered to encode phenylalanine by oligonucleotide-directed mutagenesis as described previously (24), and the resulting plasmid was used as a template in a PCR with the primers used in the construction of pJPH10 (described above). The amplified $FUS3^{Y182F}$ ORF was then ligated into pJPH11 at the *Bam*HI and *Xba*I sites to generate pJPH16. pJPH17 and pJPH19 were constructed with standard subcloning techniques using pJPH10 and plasmids derived from the genetic screen (see below).

Genetic screen for *FUS3* mutants and construction of mutant *FUS3* alleles. The PCR primers used in the construction of pJPH10 (described above) were used in PCR mutagenesis of the *FUS3* ORF as described by Leung et al. (33). These products were then digested with *Bam*HI and *Xba*I and ligated into pJPH11 at those sites. The ligation reaction was electroporated into *Escherichia coli* to yield more than 120,000 transformants. These colonies were pooled, and plasmid DNA was prepared. Control experiments showed that more than 90% of the plasmids in the library contained a single *FUS3* insert. The *fus3 kss1 far1* strain JPHY12 was then transformed with the mutant *FUS3* plasmid library and plated on selective media that contained 0.6 M NaCl. After 4 or 5 days at 30°C, sterile nylon filters were used to lift portions of each transformed colony on the plates. These membranes were frozen in liquid nitrogen, laid onto Whatman paper saturated with Z buffer (1) that contained the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) at 0.3 mg/ml, and incubated overnight at 30°C. Transformed colonies that corresponded to blue signals in this filter lift assay were then isolated. These isolates were grown on solid selective media in the presence and absence of 0.6 M NaCl and compared in the filter lift assay for salt-dependent β-galactosidase expression. Plasmid DNA was isolated from four transformants that exhibited a salt-dependent *FUS1-lacZ* expression. These plasmids were retransformed into JPHY12, and plasmid-dependent β-galactosidase expression phenotypes were confirmed as described above. The DNA sequence of each of these four mutant *FUS3* genes was compared to that of the wild-type *FUS3* ORF in pJPH10 by the dideoxy (Sanger) sequencing method using alkali denaturation of double-stranded plasmid DNA (1). The nucleotide sequence of the wild-type allele was identical to the published *FUS3* sequence (23).

Immunoblot analysis. Yeast strains were grown to a density of 1×10^7 to 2×10^7 cells per ml at 30°C in SD medium, transferred to YPD medium for 1 to 2 h, stimulated, and harvested by centrifugation at 4°C. Cells were lysed at 4°C by agitation with ~1 volume of glass beads and ~3 volumes of buffer as previously described (10). Extracts were clarified by centrifugation, mixed with an equal volume of saturated ammonium sulfate, and allowed to precipitate overnight at 4°C. The precipitates were collected by centrifugation and washed with 50% saturated ammonium sulfate that contained 1 mM EDTA, and 40 μg of each was subjected to immunoblot analysis. Electrophoresis, transfer conditions, and the antiphosphotyrosine monoclonal antibody were as described previously (2). Blocking solution for antiphosphotyrosine immunoblots was TBST (50 mM Tris [pH 8.0], 150 mM NaCl, 0.02% NaN_3, 0.05% Tween 20) containing 3% bovine serum albumin, and blocking solution for anti-Fus3p (24) and anti-Hog1p (47) immunoblots was TBST containing 5% nonfat dry milk. All blots were incubated with primary antibodies overnight at 4°C. Immunoreactivity was visualized by using a secondary alkaline phosphatase-conjugated antibody (Promega Corp.) as described previously (2). Antiphosphotyrosine immunoreactivity was quantitated by using a secondary ^{125}I-conjugated goat anti-mouse antibody (DuPont NEN) at 5×10^5 cpm/ml, exposing the immunoblots in a PhosphorImager storage screen, and quantifying the bands of interest on a Molecular Dynamics PhosphorImager (model 445SI).

Assay of β-galactosidase. β-Galactosidase activity was measured as described previously (45), with slight modifications. Briefly, strains were grown to a density of 1×10^7 to 2×10^7 cells per ml at 30°C in SD medium, then transferred to YPD for 1 to 2 h, stimulated, and harvested by centrifugation at 4°C. The cells were frozen at −80°C both before and after a continuous glass bead lysis that lasted 5 min. Approximately 50 μg of crude lysate protein was used when α-factor-treated samples were assayed, but ≥150 μg of lysate was used when unstimulated samples were assayed.

RESULTS

Osmotic stress activates Fus3p, and deletion of HOG pathway kinases increases this activation. We investigated whether the Fus3p MAP kinase could be phosphorylated on tyrosine in response to an increase in the extracellular osmolarity. To assess osmotic stress-induced changes in the phosphotyrosine content of Fus3p, it was necessary to constitutively overexpress the *FUS3* ORF because the basal expression of *FUS3* is normally quite low (22–24). A *fus3 kss1 HOG1* strain carrying *FUS3* under the control of the *GPD* promoter was exposed to 0.5 M NaCl for various times, and protein extracts were prepared. The phosphotyrosine content of Fus3p was measured by immunoblot analysis with an antiphosphotyrosine monoclonal antibody, and Fus3 protein levels were measured with an anti-Fus3p polyclonal antibody. The phosphotyrosine content of Fus3p increased approximately twofold in response to osmotic stress in the absence of any change in Fus3 protein levels (Fig. 1). This increase in the phosphotyrosine content of Fus3p was maximal within 5 min of osmotic stress. Parallel experiments were performed in an isogenic *hog1*-Δ background. In these experiments, the phosphotyrosine content of Fus3p also increased in response to osmotic stress (Fig. 1). Surprisingly, the basal phosphotyrosine content of Fus3p in the *hog1*-Δ strain was greater than that seen in the *HOG1* strain (compare the two zero time points in Fig. 1). Moreover, the osmotic stress-induced increase in the phosphotyrosine content of Fus3p was greater and continued for a longer duration in the *hog1*-Δ strain. Thus, 45 min after addition of NaCl, the phosphotyrosine content of Fus3p in a *hog1*-Δ strain was 13-fold higher than that seen in the isogenic *HOG1* strain. Identical results were obtained when 0.7 M sorbitol was used as the osmolyte (data not shown). These results show that Fus3p can be phosphorylated on tyrosine in response to increases in the extracellular osmolarity and that the *HOG1* MAP kinase can repress this osmotic stress-induced increase in Fus3p phosphotyrosine.

It has been shown by others that the phosphorylation of tyrosine 182 in Fus3p by Ste7p is required for Fus3p signalling activity and, furthermore, that Fus3p phosphotyrosine is undetectable in a mutant harboring a tyrosine 182-to-phenylalanine substitution ($FUS3^{Y182F}$) (25). To determine whether the osmotic stress-induced increase in Fus3p phosphotyrosine occurred at tyrosine 182, we performed experiments similar to those shown in Fig. 1, using a $FUS3^{Y182F}$ mutant. *fus3 kss1 HOG1* and *fus3 kss1 hog1*-Δ strains carrying *FUS3* or $FUS3^{Y182F}$ under the control of the *GPD* promoter were exposed to 0.5 M NaCl for 0 and 5 min, and protein extracts were prepared. The immunoblot analyses of these extracts are shown in Fig. 2. Phosphotyrosine was undetectable in the Fus3-Y182Fp mutant kinase under all conditions tested. In addition, we have observed that overexpressed Fus3p is not phosphorylated on tyrosine in response to osmotic stress in strains that are deleted for the mating pathway MEK *STE7* (data not shown). Thus, the osmotic stress-induced increase in tyrosine phosphorylation of Fus3p and the HOG-mediated repression of the phosphotyrosine content of Fus3p reflect changes at tyrosine 182 which are *STE7* dependent.

Experiments were done to determine whether the osmotic stress-induced tyrosine phosphorylation of Fus3p and the HOG-mediated repression of Fus3p phosphotyrosine require the HOG pathway MEK *PBS2. PBS2* and *pbs2*-Δ strains that constitutively overexpress Fus3p were exposed to 0.5 M NaCl, protein extracts were prepared, and immunoblot analyses were performed (Fig. 3). The phosphotyrosine content of Fus3p was greater in the *pbs2*-Δ strain than in the isogenic *PBS2* control. In addition, the phosphotyrosine content of Fus3p was increased by osmotic stress in the presence or absence of *PBS2*. Therefore, *PBS2* is required for the negative regulation of the phosphotyrosine content of Fus3p by the HOG pathway.

Despite the increase in the phosphotyrosine content of Fus3p, we have been unable to demonstrate an increase in mating-specific gene expression (i.e., *FUS1-lacZ* [see below]) following the addition of osmolytes to either *HOG1* or *hog1*-Δ strains cultured in liquid media. Our inability to detect these changes could be due to a nonspecific decrease in protein synthesis that

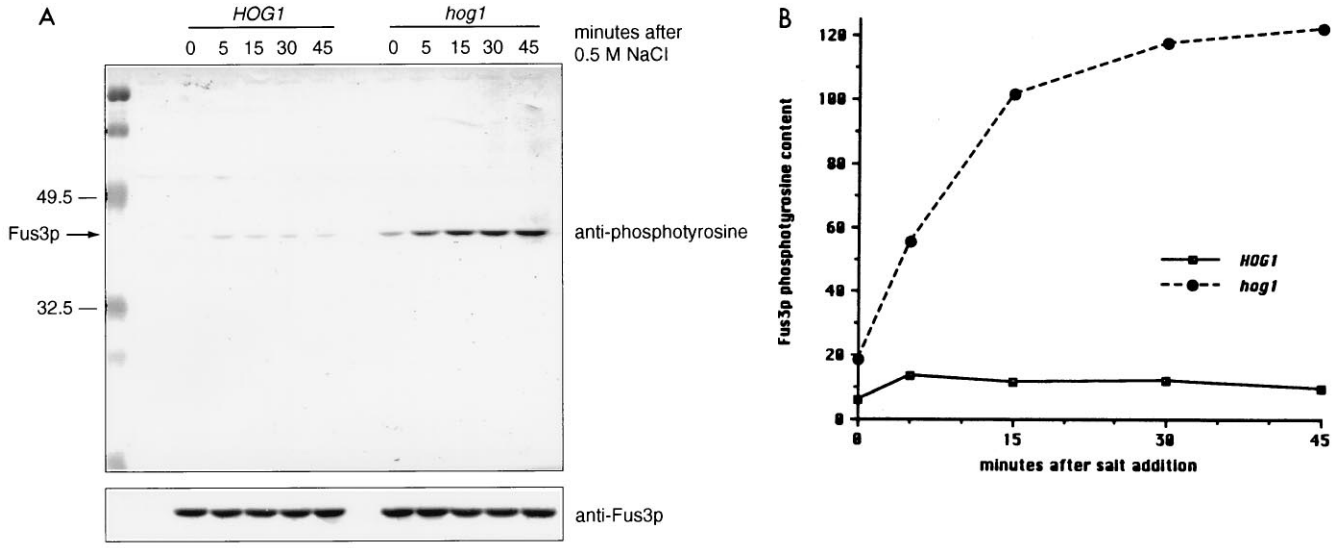

FIG. 1. Fus3p is phosphorylated on tyrosine in response to osmotic stress, and the deletion of *HOG1* increases this response. (A) Cultures of yeast strains JPHY10 (*fus3 kss1 HOG1*; left) and JPHY11 (*fus3 kss1 hog1*-Δ; right) transformed with the *FUS3* overexpression construct pJPH10 were exposed to 0.5 M NaCl for the indicated times, protein extracts were prepared, and antiphosphotyrosine and anti-Fus3p immunoblot analyses were performed. Prestained molecular weight standards and their corresponding masses in kilodaltons are indicated on the left. (B) Samples used for panel A were subjected to antiphosphotyrosine immunoblot analysis, and immunoreactivity was quantitated as described in Materials and Methods. Values shown represent arbitrary units.

follows osmotic shock and/or the relatively rapid restoration of the osmotic gradient across the plasma membrane (10).

***FUS3* expression is higher and pheromone-dependent accumulation of tyrosine-phosphorylated Fus3p is more rapid in a *hog1*-Δ background.** Expression of *FUS3* is low during vegetative growth, but it is much higher in the presence of mating pheromone (22–24). The accumulation of tyrosine-phosphorylated Fus3 protein following pheromone treatment was compared between *HOG1* and *hog1*-Δ strains in which *FUS3* expression was controlled by its normal promoter. As shown in Fig. 4, both the basal and pheromone-stimulated levels of Fus3 protein were greater in the *hog1*-Δ background. Furthermore, the phosphotyrosine content of Fus3p reached a maximum earlier in the *hog1*-Δ strain (~30 min) than in the isogenic *HOG1* strain (~45 min). Taken together, these data show that in a *hog1*-Δ strain, the mating pathway is derepressed. The higher basal level of Fus3 protein in the *hog1*-Δ strain is consistent with a repression of mating pathway activity by the HOG pathway because the *FUS3* promoter is itself pheromone responsive.

***hog1*-Δ strains are more sensitive to α-factor and have increased levels of *FUS1-lacZ* gene expression.** Mating pathway activity is often assessed by measuring the expression of the pheromone-inducible *FUS1* promoter using *FUS1-lacZ* reporter constructs (39, 54). In unstimulated cultures, the expression of a chromosomal *FUS1-lacZ* gene was more than twofold greater in a *hog1*-Δ background than in a wild-type control strain (Table 3), consistent with the effect on *FUS3* expression shown in Fig. 4. Therefore, the HOG pathway can repress the level of pheromone-responsive gene expression in unstimulated cells. In α-factor-treated cells, levels of expression of *FUS1-lacZ* were identical in wild-type and *hog1*-Δ strains. In contrast, the pheromone-responsive *FUS3* gene is expressed at higher levels in *hog1*-Δ strains both in the presence and in the absence of α-factor (Fig. 4). The reasons for this difference are unclear, and it may indicate that maximal *FUS1* expression does not require maximal mating pathway signalling activity.

Sensitivity to mating pheromone can be assessed by measuring the level of α-factor required to inhibit cell growth. As shown in Fig. 5, the growth of a *hog1*-Δ strain was inhibited at α-factor concentrations that permitted growth of the isogenic *HOG1* strain. A modest increase in the sensitivity of *hog1*-Δ strains to growth arrest by α-factor was also observed in halo assays (data not shown).

These results show that the HOG pathway-mediated repression of mating pathway activity can be observed not only at the level of Fus3p phosphotyrosine content but also at the levels of pheromone-responsive gene expression and cell growth.

The HOG pathway-mediated repression of mating pathway activity requires Hog1p catalytic activity. Lysine 52 in Hog1p resides in the putative phosphotransfer domain (V-A-I-K^{52}-K-I), and this lysine residue is completely conserved in subdo-

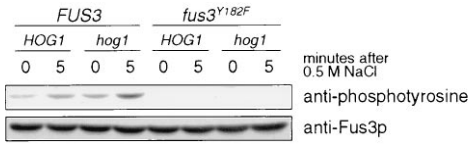

FIG. 2. The osmotic stress-induced tyrosine phosphorylation of Fus3p and the HOG-mediated repression of Fus3p tyrosine phosphorylation both occur at tyrosine 182. Cultures of yeast strains JPHY10 (*fus3 kss1 HOG1*) and JPHY11 (*fus3 kss1 hog1*-Δ) transformed with either the *wtFUS3* or *FUS3*^{Y182F} overexpression construct pJPH10 or pJPH16 were exposed to 0.5 M NaCl for 0 and 5 min, protein extracts were prepared, and antiphosphotyrosine and anti-Fus3p immunoblot analyses were performed.

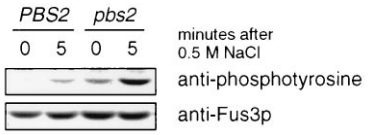

FIG. 3. The deletion of *PBS2* causes an increase in the phosphotyrosine content of Fus3p. Cultures of yeast strains YPH102a (*PBS2*) and MAY1 (*pbs2*-Δ) transformed with the *FUS3* overexpression construct pJPH10 were exposed to 0.5 M NaCl for 0 and 5 min, protein extracts were prepared, and antiphosphotyrosine and anti-Fus3p immunoblot analyses were performed.

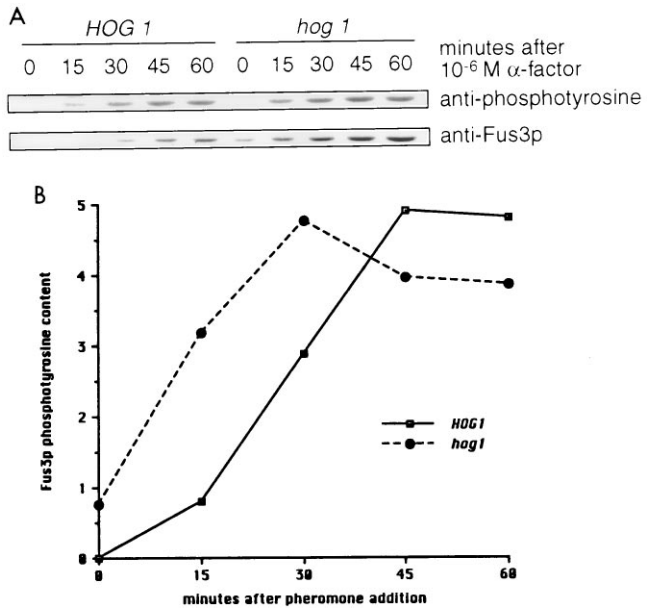

FIG. 4. *FUS3* expression is higher and the pheromone-induced accumulation of tyrosine-phosphorylated Fus3 protein is more rapid in a *hog1*-Δ background. (A) Cultures of yeast strains JPHY10 (*fus3 kss1 HOG1*; left) and JPHY11 (*fus3 kss1 hog1*-Δ; right) transformed with pJPH12, which contains the *FUS3* ORF under the control of the authentic *FUS3* promoter, were treated with 10^{-6} M α-factor for the indicated times, protein extracts were prepared, and antiphosphotyrosine and anti-Fus3p immunoblot analyses were performed. Coomassie blue-stained gels showed that identical amounts of total protein were analyzed in all samples. (B) Samples used for panel A were subjected to antiphosphotyrosine immunoblot analysis, and immunoreactivity was quantitated as described in Materials and Methods. Values shown represent arbitrary units.

main II of all known protein kinases (27). In contrast to *hog1*-Δ strains, a $hog1^{K52R}$ strain is not completely sensitive to osmotic stress. In addition, HOG pathway-inducible gene expression is weakly induced by osmotic stress in a strain harboring the $hog1^{K52R}$ allele (47). Thus, it appears that substitution of lysine 52 with arginine substantially decreases but does not abolish the catalytic activity of Hog1p. Using the $hog1^{K52R}$ allele, we tested whether the catalytic activity of Hog1p is required for the HOG-mediated repression of mating pathway activity. As shown in Fig. 6, the phosphotyrosine content of constitutively

TABLE 3. Increase in *FUS1-lacZ* expression upon deletion of *HOG1* or expression of $hog1^{K52R}$[a]

Plasmid in *fus3 kss1* strain	*hog1* allele tested	β-Galactosidase activity ratio, *hog1*/*HOG1*[b]	
		−α-factor	+α-factor
pJPH12 or pYEE81 (*FUS3*)[c]	*hog1*-Δ	2.4 ± 0.5	1.1 ± 0.1
pJPH12 (*FUS3*)[d]	$hog1^{K52R}$	1.4 ± 0.1	1.0 ± 0.1
pJPH24 ($FUS3^{D317G,F329S}$)[e]	*hog1*-Δ	2.0 ± 0.2	1.0 ± 0.1

[a] Cultures were treated with 10^{-6} M α-factor or left untreated, protein extracts were prepared, and β-galactosidase specific activities were determined as described in Materials and Methods. On average, the untreated *HOG1* strains contained 0.4 U of β-galactosidase activity and the α-factor-treated *HOG1* strains contained 15.0 U.

[b] Values are calculated from at least four independent determinations for each strain.

[c] Strain JPHY10 (*fus3 kss1 HOG1*) or JPHY11 (*fus3 kss1 hog1*-Δ) harboring the *FUS3* gene in pJPH12 or pYEE81.

[d] Strain JPHY11 harboring pJPH12 and either *HOG1* in pJPH13 or $hog1^{K52R}$ in pJPH14.

[e] Strain JPHY10 or JPHY11 harboring $FUS3^{D317G,F329S}$ in pJPH24.

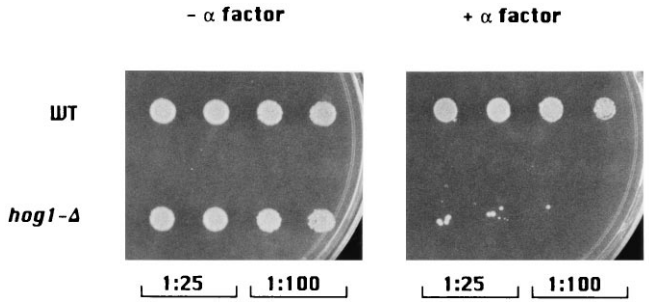

FIG. 5. *hog1*-Δ strains show increased sensitivity to growth arrest by mating pheromone. Two isolates of wild-type (WT) strain EY1118 and two isolates of the isogenic *hog1*-Δ strain CY196 were grown overnight and diluted 1:25 and 1:100 before aliquots were spotted onto solid rich medium containing or lacking 3.5 nM α-factor as indicated.

expressed Fus3p was greater in the $hog1^{K52R}$ strain than it was in the isogenic *HOG1* strain both in the presence and in the absence of pheromone. We also determined the effect of the $hog1^{K52R}$ allele on the expression of an integrated *FUS1-lacZ* reporter in *fus3 kss1* strains carrying a single-copy plasmid in which *FUS3* was under the control of its own promoter (Table 3). During vegetative growth, β-galactosidase activity was 1.4-fold greater in the $hog1^{K52R}$ strain than in the isogenic wild-type strain. Thus, HOG pathway-mediated repressions of both the phosphotyrosine content of Fus3p and the basal expression of *FUS1-lacZ* require the catalytic activity of Hog1p.

Isolation of *FUS3* alleles that allow increased *FUS1-lacZ* expression in response to high extracellular osmolarity. The high osmolarity-induced tyrosine phosphorylation of Fus3p shown in Fig. 1 suggested that *FUS3* mutants that would allow readily detectable mating-specific gene expression in response to osmotic stress might be isolated. To identify such alleles, a library of overexpressed, PCR-mutagenized *FUS3* ORFs was screened for those that allowed significant *FUS1-lacZ* expression when the cells were grown on solid medium containing 0.6 M NaCl but not when the cells were grown on solid medium alone. The yeast strain used for this was JPHY12 (*fus3 kss1 far1*) to avoid any interference of endogeneous *FUS3* and *KSS1* and to prevent osmotic stress from causing G_1 arrest and the elimination of certain *FUS3* alleles from the screen. Four of 28,000 colonies screened exhibited a salt-dependent *FUS1-lacZ* expression phenotype that was linked to the *FUS3* plasmid. In all cases, the salt-dependent expression of *FUS1-lacZ*

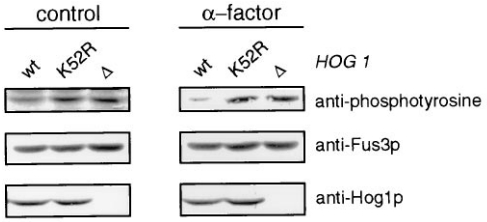

FIG. 6. The phosphotyrosine content of Fus3p is greater in a catalytically weakened *hog1* mutant. Yeast strain JPHY11 (*fus3 kss1 hog1*-Δ) harboring the constitutive *FUS3* overexpression construct pJPH10 and either the *wtHOG1* plasmid pJPH13, the $hog1^{K52R}$ plasmid pJPH14, or the *hog1*-Δ plasmid pJPH15 was treated with 10^{-6} M α-factor (α-factor) or left untreated (control). Protein extracts were prepared, and antiphosphotyrosine, anti-Fus3p, and anti-Hog1p immunoblot analyses were performed as described in Materials and Methods. The development time used to visualize the control signals in the antiphosphotyrosine immunoblot was substantially longer than the development time used to visualize the α-factor-treated signals. wt, wild type.

was observed only in colonies grown on solid media, not in those assayed following a short-term osmotic shock of yeast cells growing in liquid media.

Two classes of mutant Fus3p kinases were expected from this screen. The first class was predicted to contain kinases that possess a constitutively active phenotype. Anti-Fus3p immunoblot analyses had shown that the steady-state level of overexpressed Fus3 protein is approximately twofold greater in cells cultured on solid media containing 0.6 M NaCl than in cells grown on solid media alone (data not shown). The reason for this is unknown, but it is possibly a result of the increased cytoplasmic glycerol concentrations that arise in response to the high extracellular osmolarity. This may stabilize certain proteins to the activities of proteases whose levels increase in the stationary phase of growth (which characterizes yeast colonies grown on solid media). Thus, a Fus3p kinase that is constitutively active might provide a salt-dependent *FUS1-lacZ* expression only because it is present in greater amounts in cells grown under conditions of osmotic stress. The second class of mutant Fus3p kinases that were expected from this screen would be superactivated by osmotic stress, insensitive to the HOG-mediated repression mechanism, or both. As described below, we isolated representatives of both classes of mutants.

Isolation of a hyperactive/high-osmolarity-remedial *FUS3* mutant. Two of the four plasmids obtained from the screen contained a leucine-to-proline substitution in codon 63 (L63P substitution) of the *FUS3* gene. Yeast strain JPHY12, which constitutively overexpresses the $FUS3^{L63P}$ allele, exhibited a 15-fold increase in *FUS1-lacZ* expression compared to the wild-type *FUS3* (*wtFUS3*) allele when cultured on solid medium containing 0.6 M NaCl. This phenotype required the constitutive overexpression of the mutant (data not shown). Several lines of evidence suggest that the L63P substitution creates a kinase that is both constitutively hyperactive and unstable in cells grown in the absence of added osmolytes. During vegetative growth in liquid medium, *FUS1-lacZ* levels were at least twofold higher in strains harboring the $FUS3^{L63P}$ allele than in strains harboring *wtFUS3*. In the presence of α-factor, strains carrying the $FUS3^{L63P}$ allele showed a more than 10-fold increase in β-galactosidase activity relative to *wtFUS3*. We were unable to detect the $FUS3^{L63P}$ gene product by anti-Fus3p immunoblot analysis of cells grown on selective agar that lacked NaCl, but this mutant Fus3p was readily observed in cells grown on selective agar that contained 0.6 M NaCl (data not shown). Therefore, it is likely that the apparent salt-dependent *FUS1-lacZ* expression associated with the L63P mutation results from a hyperactive Fus3p that is preferentially stabilized in high extracellular osmolarity. The L63P mutant was not studied further, and we refer to this class of mutant as hyperactive/high-osmolarity remedial.

Isolation of a Fus3p kinase that is resistant to the HOG-mediated repression of its phosphotyrosine content. One of the two remaining mutant *FUS3* alleles encodes a Fus3 protein that allowed a twofold increase in *FUS1-lacZ* expression when cultured on solid medium containing 0.6 M NaCl, and as in the mutant described above, this phenotype required that the mutant be constitutively overexpressed. This mutant was judged not to be hyperactive in liquid β-galactosidase assays compared to wtFus3p. In addition, the levels of this mutant protein were comparable to those of wtFus3p in cells grown on solid media containing or lacking NaCl (data not shown). Therefore, it was not a likely candidate for the hyperactive/high-osmolarity-remedial class of mutant described above. DNA sequence analysis and subcloning experiments showed that two separate substitutions are required for the salt-dependent *FUS1-lacZ* expression associated with this mutant: aspartic acid 317 to glycine (D317G) and phenylalanine 329 to serine (F329S).

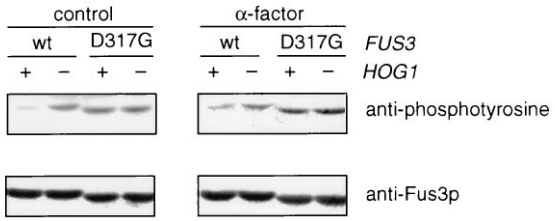

FIG. 7. Phosphotyrosine contents of a Fus3p kinase harboring a D317G substitution are identical in *HOG1* and *hog1*-Δ strains. Yeast strains JPHY10 (*fus3 kss1 HOG1*) and JPHY11 (*fus3 kss1 hog1*-Δ) were transformed with either the *wtFUS3* overexpression construct pJPH10 or the $FUS3^{D317G}$ overexpression construct pJPH17. Cultures of transformants were either treated with 10^{-6} M α-factor (α-factor) or left untreated (control), protein extracts were prepared, and antiphosphotyrosine and anti-Fus3p immunoblot analyses were performed. The development time used to visualize the control signals in the antiphosphotyrosine immunoblot was substantially longer than the development time used to visualize the α-factor-treated signals. wt, wild type.

Immunoblot analyses were performed on *fus3 kss1 HOG1* and *fus3 kss1 hog1*-Δ strains that constitutively overexpress *wtFUS3*, $FUS3^{D317G}$, $FUS3^{F329S}$, and $FUS3^{D317G,F329S}$. Surprisingly, the phosphotyrosine contents of mutant Fus3 proteins containing the aspartic acid 317 substitution were identical in the *HOG1* and *hog1*-Δ backgrounds both in the presence and in the absence of α-factor (Fig. 7). The phosphotyrosine contents of these mutant Fus3 proteins were also identical to that seen in wtFus3p in the *hog1*-Δ strain. Therefore, substitution of aspartic acid 317 with glycine renders the mutant Fus3 kinase resistant to the HOG-mediated repression of its phosphotyrosine content. Furthermore, these results suggest that the salt-dependent increase in *FUS1-lacZ* expression associated with overexpressed $FUS3^{D317G,F329S}$ requires this resistance.

Aspartic acid 317 in Fus3p is highly conserved among MAP kinases, and the corresponding residue is mutated to asparagine (N) in a gain-of-function allele of the Rolled MAP kinase in *Drosophila melanogaster* (5, 13, 56). We therefore created a $FUS3^{D317N}$ allele and assayed the phosphotyrosine content of its encoded gene product in *HOG1* and *hog1*-Δ strains. Fus3-D317Np, like Fus3-D317Gp, is resistant to the HOG-mediated repression of its phosphotyrosine content (data not shown).

In the *HOG1* and *hog1*-Δ strains, the phosphotyrosine contents of Fus3 proteins with a substitution of aspartic acid 317 were the same, but basal expression of the *FUS1-lacZ* reporter was still greater in the *hog1*-Δ background. β-Galactosidase activities were measured in *fus3 kss1 HOG1* and *fus3 kss1 hog1*-Δ strains that harbor an integrated *FUS1-lacZ* reporter and that carry a plasmid expressing either *wtFUS3* or $FUS3^{D317G,F329S}$ from the normal *FUS3* promoter (Table 3). The D317G and F329S substitutions in Fus3p had no effect on the α-factor-induced increase in *FUS1-lacZ* expression. In the absence of α-factor, however, the deletion of *HOG1* caused a similar increase in β-galactosidase activity in *wtFUS3* and $FUS3^{D317G,F329S}$ strains. These results indicate that during vegetative growth, the HOG pathway represses mating pathway activity at multiple points in the mating pheromone response pathway, including the phosphotyrosine content of Fus3p (see Discussion).

To determine whether the resistant phenotype associated with the $FUS3^{D317G,F329S}$ mutant could be observed at the level of cell growth, α-factor sensitivity assays were performed. *HOG1* and *hog1*-Δ strains expressing either *wtFUS3* or the resistant $FUS3^{D317G,F329S}$ mutant from the normal *FUS3* promoter were spotted onto selective growth media containing α-fac-

tor (Fig. 8). These data demonstrate that the *FUS3*^{D317G,F329S} mutant strain is more sensitive to mating pheromone. Furthermore, this increased sensitivity to pheromone in the *FUS3*^{D317G,F329S} strains was augmented by the deletion of *HOG1*. This latter fact is consistent with the HOG pathway repressing mating pathway activity at multiple points in the pheromone response pathway.

DISCUSSION

In the present work, we demonstrate that the osmoregulatory (HOG) pathway represses mating pathway activity and that this repression requires the catalytic activity of Hog1p. These results are consistent with the results of Stevenson et al. in which a loss-of-function mutation in *PBS2* was obtained in a screen designed to identify negative regulators of mating pathway activity (51). In a wild-type strain, Fus3p phosphotyrosine increases modestly and transiently following an increase in the extracellular osmolarity, but it increases to a greater extent and for a sustained duration in the isogenic *hog1*-Δ strain. Therefore, the HOG-mediated repression of mating pathway activity may insulate the mating pathway from activation by osmotic stress. Using a genetic screen, we isolated a *FUS3* allele that encodes a MAP kinase which is resistant to the HOG-mediated repression of its phosphotyrosine content. This kinase contains an amino acid substitution at a highly conserved aspartic acid in its DPXDEP motif. We propose, therefore, that the DPXDEP motif is necessary for the interaction of MAP kinases with factors that can negatively regulate MAP kinase activity.

The mating pathway is contacted by at least two HOG-mediated repressive effects. The increased phosphotyrosine content of Fus3p in *hog1* or *pbs2* strains suggests that the HOG pathway can exert its repressive effect on the mating pathway upstream of Fus3p, on Fus3p itself, or both. However, the phosphotyrosine contents of Fus3p kinases that contain substitutions of aspartic acid 317 are identical in *HOG1* and *hog1*-Δ strains (Fig. 7). If the repression mechanism were to act on an upstream activator of Fus3p, these mutants could be explained only by an insensitivity to that activator. Although this remains a possibility, we view it as unlikely given that strains harboring the resistant *FUS3*^{D317G,F329S} alleles can respond normally to α-factor (Table 3 and data not shown). For these reasons, we feel that a HOG-mediated repressive effect alters Fus3p directly and thereby changes its phosphotyrosine content.

We have shown that basal expression of the *FUS1-lacZ* reporter gene is repressed by the HOG pathway (and that this repression requires the catalytic activity of Hog1p [Table 3]). This effect was also observed in strains expressing the resistant *FUS3*^{D317G,F329S} and *FUS3*^{D317G} alleles (Table 3 and data not shown). In addition, a *hog1*-Δ *FUS3*^{D317G,F329S} strain is more sensitive to growth arrest by α-factor than the isogenic *HOG1* *FUS3*^{D317G,F329S} strain (Fig. 8). From these data, we conclude that the HOG pathway must repress the mating pathway by at least one mechanism that does not alter the phosphotyrosine content of Fus3p. These repressive effects may act upstream of Fus3p, at the level of Fus3p, or downstream of Fus3p. These three possibilities are not mutually exclusive, and at this point none of them can be ruled out.

It is possible that a MAP kinase-specific phosphatase is activated by Hog1p and is responsible for the HOG-mediated repression of Fus3p phosphotyrosine (52). Msg5p is somewhat specific for Fus3p, and Ptp2p is presumably specific for Hog1p (21, 36). We tested for the involvement of both Msg5p and Ptp2p in the repression mechanism by using antiphosphotyrosine immunoblot analysis. The deletion of either *MSG5* or *PTP2* had no effect on the HOG-mediated repression of the phosphotyrosine content of Fus3p (data not shown). However, we note that there are additional protein tyrosine phosphatases with strong similarities to *MSG5* and *PTP2* that have been identified through the yeast genome sequencing project. It is possible that one or more of these phosphatases plays such a role.

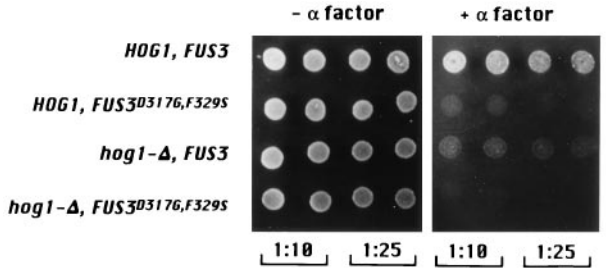

FIG. 8. *FUS3*^{D317G,F329S} and *hog1*-Δ strains show increased sensitivity to growth arrest by mating pheromone. Cultures of yeast strains JPHY10 (*fus3 kss1 HOG1*) and JPHY11 (*fus3 kss1 hog1*-Δ) were transformed with either the single-copy *wtFUS3* or *FUS3*^{D317G,F329S} expression construct pJPH12 or pJPH24. In both of these plasmids, the *FUS3* ORF is under the control of the authentic *FUS3* promoter. Transformants were grown overnight and diluted 1:10 and 1:25 before aliquots were spotted onto solid minimal media lacking uracil and containing or lacking 25 nM α-factor.

The Ste5 protein interacts with multiple kinases in the mating cascade (16, 37, 42), and it has been shown to function as a scaffold that allows Ste11p, Ste7p, Fus3p, and Kss1p to form a multikinase complex (16). This complex could permit the sequential phosphorylations in the MAP kinase module to occur more efficiently and may provide an added level of specificity to these reactions. Consistent with the latter hypothesis, Yashar et al. have recently shown that a hyperactive Ste7p variant can compensate for defects in the cell integrity (i.e., *MPK1*) pathway in a *ste5* background (57).

One explanation for the HOG-mediated repressions of mating pathway activity described here involves a competition between Hog1p and Fus3p for binding to a Ste5p-mediated multikinase complex. However, HOG-mediated repression of the mating pathway is observed in strains expressing Fus3p from the authentic *FUS3* promoter (Fig. 4, 5, and 8 and Table 3) as well as in strains overexpressing Fus3p (Fig. 1, 2, 3, 6, and 7). If a stoichiometric competition exists between Hog1p and Fus3p for a binding site in the Ste5p complex, then in the latter set of experiments, the overexpression of Fus3p should have reduced the occupancy of endogenous Hog1p in that complex. Furthermore, the increased mating pathway activation seen in *pbs2*-Δ and *hog1*^{K52R} strains also argues against the repression being a stoichiometric competition between Fus3p and Hog1p.

The HOG-mediated repression of mating pathway activity may serve an insulation function. The existence of multiple MAP kinase modules that function in a single cell type implies that the activation of a given module must be tightly controlled and highly specified in order to avoid indiscriminate activations that could lead to inappropriate cellular responses. A related consideration has long been recognized in bacteria which simultaneously express multiple two-component (histidine kinase) signalling modules. In these systems, there is evidence to suggest that activation of a given pathway can lead to weak activation of parallel pathways that may be physiologically unrelated. The term "cross talk" was chosen to refer to such presumably nonspecific activations (55). We propose that the weak activation of the mating pathway by an osmotic stimulus

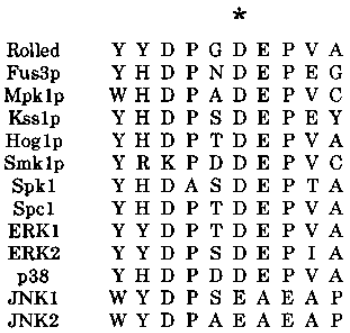

FIG. 9. Alignment of DPXDEP motifs from MAP kinases in *D. melanogaster*, yeasts, and humans. Rolled is from *D. melanogaster* (6); Fus3p (23), Mpk1p (32), Kss1p (18), Hog1p (10), and Smk1p (31) are from *S. cerevisiae*; Spk1 (53) and Spc1 (48) are from *Schizosaccharomyces pombe*; ERK1 (9), ERK2 (8), p38 (26), JNK1 (20), and JNK2 (29) are from humans.

may also represent nonspecific interactions and therefore may be viewed as cross talk as defined above. It has also been recognized that a given signalling pathway can bifurcate to activate multiple pathways and that regulatory mechanisms exist between pathways to achieve appropriate activation states and appropriate physiological responses. In bacterial systems, the term "cross-regulation" was chosen to refer to this class of interpathway interactions (55). The term "insulation" has been used by Bardwell et al. (3) to describe mechanisms that prevent the adventitious activation of one signalling pathway by another. We propose that the HOG-mediated repression of the mating pheromone response pathway may represent a cross-regulatory mechanism that insulates the mating pathway from nonspecific cross talk following cellular stimulation by high extracellular osmolarity. The greater osmotic stress-induced increase in Fus3p phosphotyrosine observed in a *hog1*-Δ mutant is consistent with this proposal (Fig. 1). The G_1 arrest and differentiation that follow mating pathway activation suggest that such cross-regulatory control mechanisms may be particularly important for maintaining a subcritical level of mating pathway activity so that a heterologous stimulus such as osmotic stress does not initiate a mating response. If this is the case, then the HOG-mediated repressive effects serve a bona fide cross-regulatory insulation function.

The resistant phenotype of *FUS3^{D317G}* mutants suggests similar repression mechanisms in other organisms. Substitution of aspartic acid 317 renders Fus3p resistant to the HOG-mediated repression of its phosphotyrosine content (Fig. 7). This finding suggests that aspartic acid 317 in Fus3p is required for the HOG-mediated repression mechanism. Figure 9 shows a comparison of the residues that surround this aspartic acid in MAP kinases from a variety of organisms. The consensus motif is DPXDEP, and it lies in the carboxy-terminal end of subdomain XI. If the HOG-mediated repression of Fus3p phosphotyrosine is mediated by a specific protein (e.g., a phosphatase), this protein may recognize the DPNDEP motif or a site in Fus3p which is altered upon substitution of aspartic acid 317.

It is possible that the resistance of these mutants can help explain MAP kinase mutants such as the sevenmaker gain-of-function allele of the *Drosophila* MAP kinase encoded by the *rolled* locus (5, 13, 56). Development of the R7 photoreceptor cell in ommatidia of the *Drosophila* eye requires an intact signalling pathway that utilizes the Sevenless receptor tyrosine kinase. When Sevenless binds to its ligand Bride-of-sevenless (Boss), a MAP kinase signalling pathway is activated which requires Rolled. In the absence of *sevenless* or *boss*, development of the R7 photoreceptor cell is rescued if aspartic acid 334 in Rolled is substituted with asparagine (13). Aspartic acid 334 in Rolled is equivalent to aspartic acid 317 in Fus3p. We speculate, therefore, that Rolled(D334N) is a gain-of-function mutant because it is resistant to a repression mechanism analogous to the HOG-mediated repression of the phosphotyrosine content of Fus3p.

The unique phenotypes associated with substitutions in the DPXDEP motif of both *Drosophila* and yeast MAP kinases point to the importance of this motif in the function of these enzymes. These phenotypes suggest that the DPXDEP motif is part of a mechanism that coordinates the interaction of MAP kinases with factors that dampen their activity, and also that the HOG-mediated repression of Fus3p phosphotyrosine may represent one example of a repression mechanism that exists among MAP kinase pathways in higher eukaryotes.

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